Protein and Lipid Quality on Protein Digestibility in Pacific White Shrimp

(*Litopenaeus vannamei*)

Duangrat Chookird

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Author	Mrs. Duangrat Chookird
Major Program	Aquatic Science

Major Advisor :

……

(Asst. Prof. Dr.Chutima Tantikitti)

Co-advisor :

……

(Prof. Dr.Amornrat Phongdara)

Examining Committee :

……....Chairperson

(Assoc. Prof. Dr.Wutiporn Phromkunthong)

……

(Asst. Prof. Dr.Chutima Tantikitti)

……

(Prof. Dr.Amornrat Phongdara)

……

(Dr.Ian Forster)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Aquatic Science

……

(Prof. Dr.Amornrat Phongdara)

Dean of Graduate School

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Author	Mrs. Duangrat Chookird
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ABSTRACT

Fishmeal and fish oil play a role as important feed ingredients in aquatic feed. However, rising demand while production has leveled off has caused a search for alternative protein and oil sources. The aim of this thesis is to test protein and lipid quality on protein digestibility in Pacific white shrimp (*L. vannamei*) including growth performance, feed utilization efficiency and trypsin gene expression.

Experiment 1 was carried out to test different grades of fishmeal. The results showed that fishmeal produced from varying raw materials resulted in different digestibility regardless of fishmeal grade classified using proximate composition. Good essential amino acid profile premium grade fishmeal (S1) produced from whole fish of single species had a greater *in-vivo* protein digestibility than those of the mixed species premium grade fishmeal (S2) and particularly grade 2 fishmeal produced from surimi processing by-product which had the highest *in-vitro* protein digestibility. The premium grade fishmeal (S1) gave the highest growth performance and feed utilization efficiency especially protein productive value in comparison with the others except imported fishmeal (Chile). Trypsin gene expression was related to the ratio of EAA/NEAA in the diets and the premium grade fishmeal (S1) showed the highest trypsin gene expression. The premium grade fishmeal was chosen and used as a main protein source in Experiment 2 to test protein digestibility of diets containing hemoglobin powder replacing fishmeal protein at 0%, 12.5%, 25%, 50%, 75% and 100%. The results showed that protein digestibility increased with increasing hemoglobin levels. Growth performance and feed utilization efficiency decreased with increasing hemoglobin levels. Trypsin gene expression was not related to the protein quality in this experiment. Experiment 3 was conducted to investigate effects of replacing fishmeal with soybean meal and hemoglobin powder on protein digestibility.

The basal diet contained fishmeal and soybean meal at 60:40. Other diets substituted fishmeal with a combination of protein at 6.73%, 13.52%, 19.80%, 19.80% + amino acid and 26.53% + amino acid. A reference diet with fishmeal as a sole source of protein was also included. Results showed that protein digestibility was the highest in the reference diet and decreased with increasing hemoglobin levels. Growth performance and feed utilization efficiency decreased with increasing hemoglobin levels. A crystalline amino acid supplementation did not improve shrimp growth but resulted in slight improvement in feed utilization efficiency at 19.80% hemoglobin. Trypsin gene expression was not related to protein quality in this experiment. The 6.73% hemoglobin substituted diet which had growth performance close to the control diet was chosen for Experiment 4.

Experiment 4 was carried out to study protein sparing effect and effects of lipid quality on protein digestibility. Factorial design with three factors, lipid levels (8% and 12%), lipid sources (fish oil and soybean oil) and protein levels (35% and 40%) was performed. Results showed that there was a protein sparing effect of fish oil diet from carbohydrate through energy balance of the diets but was not by lipid levels. Protein digestibility of diet at 8% lipid was higher than 12% lipid. Soybean oil diets gave higher protein digestibility, growth performance and feed utilization efficiency and those at 8% soybean oil was better than 12% soybean oil excluding the best growth of 8% fish oil with 35% protein diet which resulted from protein sparing effects. Relationship of lipid quality and trypsin gene expression was not found.

ชื่อวิทยานิพนธ์ คุณภาพโปรตีนและไขมันต่อประสิทธิภาพการย่อยโปรตีนในกุ้งขาว (*Litopenaeus vannamei*)

ผู้เขียน นางดวงรัตน์ ชูเกิด

สาขาวิชา วาริชศาสตร์

ปีการศึกษา 2553

บทคัดย่อ

ปลาป่นและน้ำมันปลาเป็นวัตถุดิบอาหารสัตว์ที่มีความสำคัญในอาหารสัตว์น้ำ เนื่องจากเป็นแหล่งโปรตีนและไขมันที่จำเป็นที่มีคุณภาพดีแต่ความต้องการที่สูงขึ้นในขณะที่การผลิตค่อนข้างจำกัดจึงจำเป็นต้องแสวงหาทางเลือกในการใช้โปรตีนและไขมันทดแทนจากแหล่งอื่น การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาคุณภาพโปรตีนและไขมันที่มีผลต่อประสิทธิภาพการย่อยโปรตีนในกุ้งขาว (*L. vannamei*) รวมทั้งการเจริญเติบโต ประสิทธิภาพการใช้อาหารและการแสดงออกของยีนส์ทริปซิน

การทดลองที่ 1 ศึกษาคุณภาพปลาป่นจากแหล่งผลิตที่ต่างกัน ผลการทดลองพบว่าปลาป่นที่ผลิตจากวัตถุดิบที่แตกต่างกันให้ประสิทธิภาพการย่อยโปรตีนที่แตกต่างกันโดยไม่ขึ้นอยู่กับระดับคุณภาพของปลาป่นซึ่งกำหนดโดยใช้องค์ประกอบทางเคมี ปลาป่นคุณภาพพรีเมี่ยมเกรดชนิด S1 ซึ่งผลิตจากปลาชนิดเดียวและเป็นปลาทั้งตัวมีประสิทธิภาพการย่อยโปรตีนสูงกว่าปลาป่นพรีเมี่ยมเกรดชนิด S2 ซึ่งผลิตจากปลาเบญจพรรณ ปลาป่นเกรด 2 ซึ่งผลิตจากวัสดุเศษเหลือจากโรงงานผลิตซูริมิมีประสิทธิภาพการย่อยที่ต่างจากชนิดอื่นๆ โดยพบว่าประสิทธิภาพย่อยโปรตีนสูงสุดจากการประเมินโดยเทคนิคการย่อยในหลอดทดลอง (*in-vitro* digestibility) ขณะที่ประสิทธิภาพการย่อยโปรตีนต่ำสุดจากการประเมินโดยใช้สัตว์ทดลอง (*in-vivo* digestibility) ปลาป่นพรีเมี่ยมเกรดชนิด S1 ให้การเจริญเติบโตและประสิทธิภาพการใช้อาหารโดยเฉพาะโปรตีนที่นำไปใช้ประโยชน์ (protein productive value) สูงที่สุดแต่ไม่แตกต่างทางสถิติกับปลาป่นชนิดอื่นๆ ยกเว้นปลาป่นนำเข้าจากต่างประเทศ (ชิลี) ปลาป่นพรีเมี่ยมเกรดชนิด S1 ให้การแสดงออกของยีนส์ทริปซินสูงที่สุด โดยการแสดงออกของยีนส์สอดคล้องกับสัดส่วนของกรดอะมิโนที่จำเป็นต่อกรดอะมิโนที่ไม่จำเป็น (EAA/NEAA) จากนั้นคัดเลือกปลาป่นพรีเมี่ยมเกรดชนิด S1 เป็นแหล่งโปรตีนสำหรับการทดลองที่ 2 ซึ่งมีวัตถุประสงค์เพื่อศึกษาการทดแทนโปรตีนในปลาป่นด้วยฮีโมโกลบินป่นที่ร้อยละ 0, 12.5, 25, 50, 75 และ 100 ผลการทดลองพบว่าประสิทธิภาพการย่อยโปรตีนเพิ่มขึ้นตามปริมาณฮีโมโกลบินที่เพิ่มขึ้น การเจริญเติบโตและประสิทธิภาพการใช้อาหาร

ลดลงตามปริมาณฮีโมโกลบินที่เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ การแสดงออกของยีนส์ทริปซินไม่สัมพันธ์กับคุณภาพโปรตีน การทดลองที่ 3 มีวัตถุประสงค์เพื่อศึกษาการทดแทนที่ปลาป่นด้วยถั่วเหลืองป่นและฮีโมโกลบินป่นโดยแทนที่โปรตีนปลาป่นด้วยถั่วเหลืองป่นที่ระดับ 60:40 เป็นสูตรพื้นฐานและทดแทนโปรตีนของปลาป่นด้วยฮีโมโกลบินป่นที่ร้อยละ 0, 6.73, 13.52, 19.80, 19.80 ร่วมกับเสริมกรดอะมิโน และ 26.53 ร่วมกับเสริมกรดอะมิโน ส่วนสูตรอ้างอิงใช้ปลาป่นเป็นโปรตีนหลัก ผลการทดลองพบว่าประสิทธิภาพการย่อยโปรตีนของทุกชุดการทดลองต่ำกว่าสูตรอ้างอิงและมีแนวโน้มลดลงเมื่อปริมาณฮีโมโกลบินเพิ่มขึ้น การเจริญเติบโตและประสิทธิภาพการใช้อาหารลดลงเมื่อปริมาณการแทนที่ด้วยฮีโมโกลบินเพิ่มขึ้น การเสริมกรดอะมิโนไม่ช่วยเพิ่มการเจริญเติบโตแต่ช่วยปรับปรุงประสิทธิภาพการใช้อาหารเล็กน้อยที่ระดับการทดแทนที่ร้อยละ 19.80 การเสริมกรดอะมิโนในระดับที่สูงขึ้นไม่สามารถช่วยปรับปรุงทั้งการเจริญเติบโตและประสิทธิภาพการใช้อาหาร โดยการแสดงออกของยีนส์ทริปซินไม่สัมพันธ์กับคุณภาพของโปรตีน จากนั้นคัดเลือกชุดการทดลองที่มีการแทนที่ด้วยฮีโมโกลบินที่ร้อยละ 6.73 ซึ่งมีการเจริญเติบโตใกล้เคียงกับชุดควบคุมเพื่อใช้ในการทดลองที่ 4

การทดลองที่ 4 มีวัตถุประสงค์เพื่อศึกษาการสำรองโปรตีน (protein sparing effect) และคุณภาพไขมัน โดยวางแผนการทดลองแบบแฟคทอเรียลซึ่งมี 3 ปัจจัยคือ ระดับไขมัน (ร้อยละ 8 และ 12), ชนิดน้ำมัน (น้ำมันปลาและน้ำมันถั่วเหลือง) และระดับโปรตีน (ร้อยละ 35 และ 40) พบว่ามีการสำรองโปรตีนโดยคาร์โบไฮเดรตของชุดการทดลองที่ใช้น้ำมันปลาเป็นแหล่งไขมัน ซึ่งเกิดจากการใช้แป้งในการปรับพลังงานในอาหารแต่ไม่พบการสำรองโปรตีนจากไขมัน สำหรับประสิทธิภาพการย่อยโปรตีนพบว่าอาหารที่มีระดับไขมันร้อยละ 8 มีประสิทธิภาพการย่อยโปรตีนที่สูงกว่าที่ระดับไขมัน 12% ขณะที่น้ำมันพืชให้ประสิทธิภาพการย่อยโปรตีน การเจริญเติบโต และประสิทธิภาพการใช้อาหารดีกว่าน้ำมันปลาโดยที่ระดับไขมันร้อยละ 8 ให้ผลดีกว่าที่ร้อยละ 12 ยกเว้นการใช้น้ำมันปลาที่ร้อยละ 8 ร่วมกับการใช้โปรตีนที่ร้อยละ 35 ให้การเจริญเติบโตดีที่สุดซึ่งเกิดจากการสำรองโปรตีน การแสดงออกของยีนส์ทริปซินไม่สอดคล้องกับคุณภาพไขมัน

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CHAPTER 1

Introduction

Diet is an important factor influencing shrimp production. Good quality diets provide good growth, whereas low quality diets not only impair growth but also negatively impact the culture water which will have a direct effect on shrimp culture. Protein is an expensive ingredient accounting for more than 50% of diet cost. In general, low quality protein, digestibility and bio-availability are a cause of organic waste from unutilized protein and amino acid into cultured environment resulting in ammonia, nitrate and nitrite increased which deteriorate water quality. These also cause of economic loss due to un-efficiency protein utilization and water treatment.

Any protein sources have varying property and benefit for cultured animal at various levels due to their quality. Good protein quality providing amino acid to meet requirement of shrimp through protein digestibility and the providing amino acids can be proper to the amino acid usage by protein retention or muscle growth. Some of absorbed amino acid may turn into energy and some are used for others amino acid containing molecule in cell. Attempting to use amino acid for muscle growth at possibly the highest is the goal for protein efficiency usage and economic for business. Quality of protein can be judged based on amino acid composition and protein utilization efficiency (PER and PPV) (Fennema, 1996). Marine protein sources especially fishmeals are the main protein sources in commercial shrimp diets due to their complete amino acid profile, high digestibly and good palatability. Quality of fishmeal is variety based on its raw material. Fishmeals produced from whole fish have higher protein and lower ash content compared to those produced from by-product of surimi processing (D'Abramo *et al*., 1997). Moreover, amino acid profile of fishmeal is also very dependent upon fishmeal sources. Herring meal containing 78.3% protein has arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine at 5.02, 1.80, 3.41, 5.64, 5.83, 2.27, 2.94, 3.16, 0.83 and 4.68 % of protein whereas that of menhaden meal was 4.09, 1.58, 3.15, 4.89, 5.15, 1.91, 2.69, 2.73, 0.71 and 3.52%, respectively (Halver and Hardy, 2002). Fishmeal protein was reported as a premiere protein source fed to shrimp (Suarez *et al*., 2009; Goytortua-Bores

et al., 2006; Forster *et al*., 2003) and fish (Uyan *et al*., 2006; Dupont-Nivet *et al*., 2009; Viola *et al*., 1982).

Demanding for fishmeal go up both of the best quality fishmeal for human consumption and consecutive lower quality fishmeal for feed. The risen demand together with uncertainty production due to the production cost and effect of environmental problem such as El Nino make demand over supply come up with the higher price and tend to be shortage in the future. To solving previous problem, fishmeal substitution with cheaper and more available protein source is a potential strategy. Soybean meal, meat and bone meal, feather meal hydrolyzed and by-product from terrestrial animal processing can be used as replacer for fishmeal. Substituting fishmeal with a single protein source is quite limiting due to the amino acid imbalance, particularly at high level replacement. A good combination of different protein sources is a solution to achieve a balance of essential amino acids. For example, combination of barley-based fermented grains and wheat gluten at 1:1 can replace fishmeal at 66% fed to *Litopenaeus vannamei* (Molina-Poveda and Morales, 2004). However, amino acid supplementation is necessary where some essential amino acid is below the requirements. Floreto *et al*. (2000) had success with amino acid supplementation to improve American lobster fed 50% soybean meal diet replacing fishmeal without promised growth. Hemoglobin powder is a feedstuff with a high protein content and digestibility and also has binding property. Therefore, it has a potential as protein source for fishmeal substitution. However, the poor cystine, methionine and isoleucine content of the meal need serious attention and consideration on mixture with other protein sources in order to have amino acid balance may be the option.

Lipid is the second most important macro nutrient for aquatic animal as sources of energy and essential fatty acids, carriers of fat soluble vitamins and required for crucial biochemical processes (De Silva and Anderson, 1995). Main lipid source used in aquatic feed is fish oil but uncertain availability has led to a search for alternative sources. The species being studied were tilapia (Huang *et al*., 1998; Souza *et al*., 2008), rainbow trout (Caballero *et al*., 2002; Richard *et al*., 2006; Figueiredo-Silva *et al*., 2005; Bureau *et al*., 2008), common carp (Yilmaz and Genc, 2006), Atlantic cod (Morkore *et al*., 2007; Pike, 2008), murray cod (Francis *et al*., 2006), Atlantic salmon (Ng *et al*., 2004; Wagner *et al*., 2004; Toledo, 2008), European sea

bass (Morais *et al*., 2007), gilthead seabream (El-kerdawy and Salama, 1997; Wassef *et al*., 2009), red seabream (Komilus, 2008), black seabream (Peng *et al*., 2008), sharpsnout seabream (Almaida-Pagan *et al*., 2007), red drum (Leffler *et al*., 2008) Kona kampachi (Maccomas *et al*., 2008), tropical gar (Contreras-Scarlos *et al*., 2008), yellowtail (Aoki *et al*., 2008), Pacific threadfin (Forster *et al*., 2008), turbot (Regost *et al*., 2003), European eel (Luzzana *et al*., 2003) and shrimp (Patnaik *et al*., 2006). The alternative oil source for fish oil investigated include palm oil products (crude palm oil, crude palm kernel oil, palm fatty acid distillates), soybean oil, sunflower oil, linseed oil, rapeseed oil, canola oil, soy acid oil, yellow grease, flaxseed oil, triolein, coconut oil, lard, beef tallow, poultry fat, oil from thraustochytrid and algae (Huang *et al*., 1998; Souza *et al*., 2008; Caballero *et al*., 2002; Richard *et al*., 2006; Figueiredo-Silva *et al*., 2005; Bureau *et al*., 2008; Richard *et al*., 2006; Yilmaz and Genc, 2006; Pike, 2008; Francis *et al*., 2006; Ng *et al*., 2004; Wagner *et al*., 2004; Toledo, 2008; Morais *et al*., 2007; El-kerdawy and Salama, 1997; Komilus, 2008; Peng *et al*., 2008; Almaida-Pagan *et al*., 2007; Leffler *et al*., 2008; Maccomas *et al*., 2008; Contreras-Scarlos *et al*., 2008; Aoki *et al*., 2008; Forster *et al*., 2008; Regost *et al*., 2003; Luzzana *et al*., 2003; Patnaik *et al*., 2006).

Every lipid source has a unique physical property such as fish oil which has good palatability and plenty of essential fatty acids especially n-3 HUFA whereas vegetable oil contains mainly n-6. Hertrampf and Piedad-Pascual (2000) addressed the lipid requirement of fish ranging from 5-18% depending on fish species but diets for Atlantic salmon (*Salmo salar*) may contain as high as 25% lipid whereas the recommendations for marine shrimp are 4-10% lipid. Peng *et al*. (2008) recommended 60-80% replacement of fish oil by soybean oil in diet for black seabream while complete substitution reduced growth efficiency. Figueiredo-Silva *et al*. (2005) reported that both European sea bass and rainbow trout can be fed diets containing up to 50% soybean oil replacing for fish oil without adverse effects on tissue lipid composition or liver histology. El-kerdawy and Salama (1997) reported that fingerling gilthead bream fed fishmeal-based control diet containing 9% fish oil had the best growth and survival, followed by fish fed 50% soybean oil, 50% linseed oil and 50% rapeseed oil diets with fish oil replacement. Patnaik *et al*. (2006) reported co-extruded soybean and poultry by-product meal and oil from heterotrophic microalgal fermentation source can be potential candidate for fishmeal and marine oil

replacement in shrimp diets with no fishmeal. Komilus (2008) reported that diets with dietary soy oil replacing fish oil fed to juvenile red seabream gave a decreased growth when inclusion levels increased, however, at 40% fish oil replacement produced optimal growth.

Dietary lipid influences protein utilization through its protein sparing action. Increase in dietary lipid level in diets from 6-18% has been shown promoting growth in red tilapia (De Silva *et al*., 1991). In contradictory protein sparing effect due to carbohydrate was observed in *P. japonicus* (Teshima and Kanazawa, 1984). Direct relationship between lipid quality and protein digestibility was not reported over supply of dietary lipid may have an effect on amino acid absorption. Morais *et al*. (2005) found that Senegalese sole (*Solea senegalensis* Kaup 1858) larvae fed soy oil emulsion enriched Artemia showed a delay in amino acid absorption coinciding with fatty acid accumulation in enterocytes also affected fatty acid absorption efficiency of lipids. The enrichment also resulted in impaired growth. Ballesta *et al*. (1991) reported an effect of lipid quality on protein digestibility in adult dogs that were fed olive oil showing improved protein digestion and metabolic utilization as compared to sunflower oil. Bureau *et al*. (2008) reported that replacing half of fish oil with beef tallow resulted in lower n-3 PUFAs in fish fillet but did not affect nutrient digestibility or growth performance of rainbow trout. Due to unclear information about relationship of protein quality and lipid quality on protein digestibility which associated with growth performance, feed utilization and trypsin gene expression in *L. vannamei*, this thesis was conducted under condition of protein substitution and protein sparing effect.

Objectives

1. To study fishmeal quality on growth performance, apparent crude protein digestibility and trypsin gene expression
2. To study hemoglobin powder substituted for fishmeal on growth performance, apparent crude protein digestibility and trypsin gene expression
3. To study fishmeal substituted with soybean meal and hemoglobin powder on growth performance, apparent crude protein digestibility and trypsin gene expression

4. To study protein sparing effect and lipid quality on growth performance, apparent crude protein digestibility and trypsin gene expression

CHAPTER 2

Review of literature

2.1 Shrimp digestive system

Digestive process in shrimp is very short which begin at mouth until pass to stomach by mechanical digestion providing smaller particle of intake diets. Fine particles are filtered and passed to midgut gland (hepatopancreas) where digestion occurs mainly by action of trypsin enzyme and chymotrypsin (D'Abramo *et al*., 1997) produced and secreted from B-cell followed with absorption and storage at R-cell (Figures 1 and 2). Undigested diets are transported through midgut and then pushed into hindgut where mucus glands produce mucus and cover feces before excreted to environment.

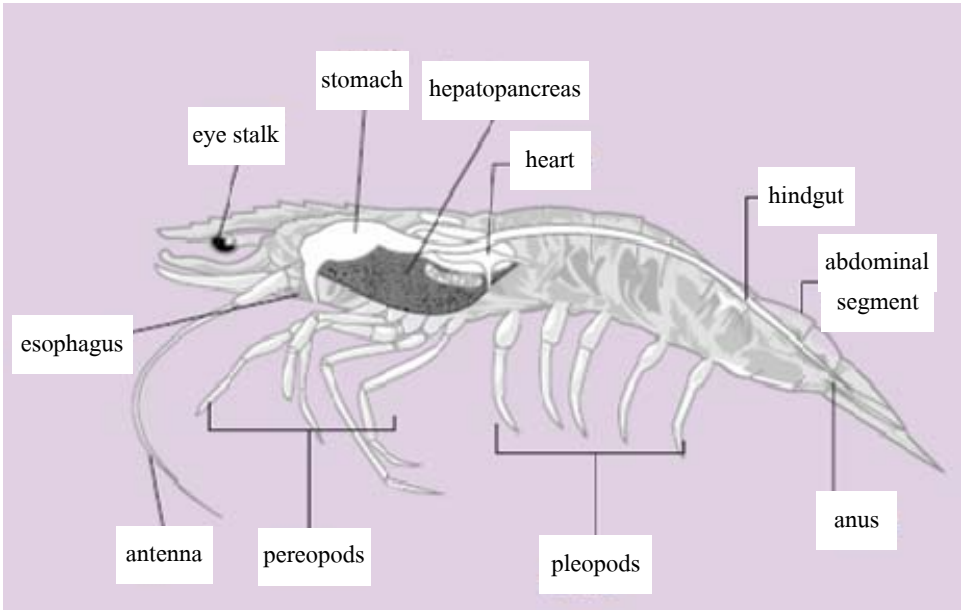
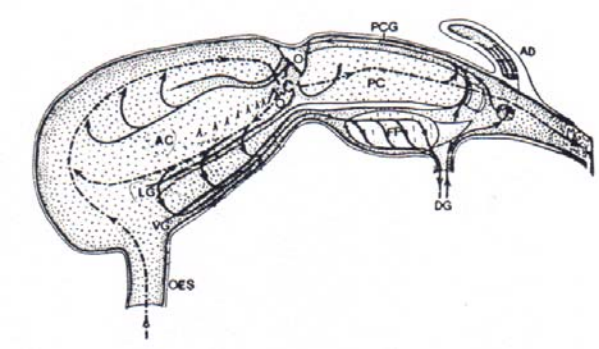


Figure 1 External and internal anatomy of penaeid shrimp (FAO and NACA, 2001)



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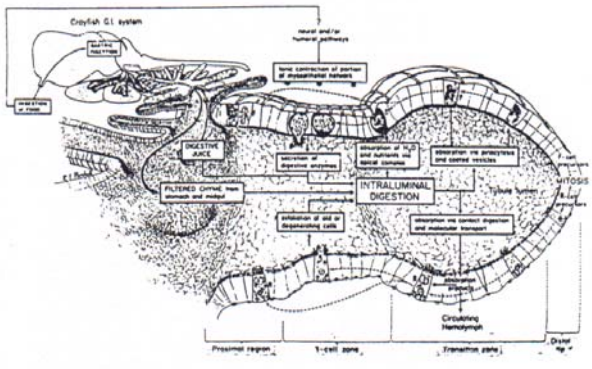

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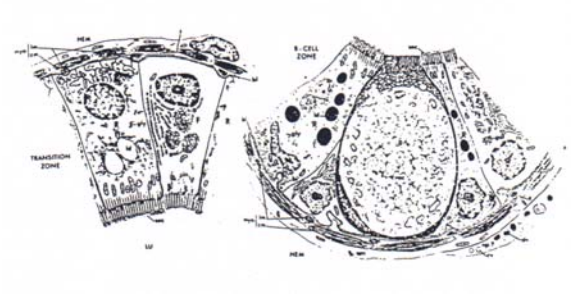

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Figure 2 Morphology of organ involving digestive system, a) digestive gland with fluid performing in proventriculus, b) blind end diverticulum showing cell variety which performed enzyme secretion and absorption, c) diagram of transition zone and B-cell zone in digestive gland showing detail of B-, F- (produced enzyme) and R-cell type (stored absorbed nutrient). (Mentel, 1983 and Loizzi, 1971 cited by Lumubol, 1995)

2.2 Protein digestion and amino acid absorption

During maintaining of ingested diets in digestive tract, digestion and absorption process of nutrients took place along with the passing of undigested items through the digestive tract until being push out into the environment as feces. Invertebrate was known to adjust their gut passage dynamics. Gut passage time (GPT) in *L. vannamei* ranged from 48.3-66.6 min (Beseres *et al*., 2005). Gut passage time was related with digestibility of nutrients including protein because the length of time food remains in the gut can influence its digestibility (portion absorbed). Previous research among different shrimp species using different experimental methods have provided conflicting results concerning how digestibility and GPT change with food quality (Lee, 1971; Sedgwick, 1979; Fair *et al*., 1980; Koshio *et al*., 1993; Stephen, 2001, Glencross *et al*., 2002). Increased GPT maximized energy uptake, perhaps causing changes in assimilation efficiency and growth rate (Taghon, 1981; Ahrens *et al*., 2001).

Protein digestion in shrimp is mainly by trypsin action and chymotrypsin to a lesser extent (D'Abramo *et al*., 1997). An adaptation of activity of digestive enzymes to the composition of diets has been found in *Palaemon serratus* (Van Wormhoudt *et al*., 1980) and *Homarus gammarus* (Lucien-Brun *et al*., 1985). Maximal specific protease activity of Palaemon serratus is reach when fed diet containing 45% protein (Van Wormhoudt *et al*., 1980). The specific digestive protease corroborate with a better growth when fed diet containing 36.6% protein. In *L. vannamei*, Lee *et al*. (1984) found that the activity of proteolytic enzymes increased as protein quality and level increased while in *P. setiferus* an inverse relationship between protein level and enzyme activity was observed (Lee and Lawrence, 1985). Le Moullac *et al*. (1994) showed that trypsin activity in *L. vannamei* larvae increased with increasing levels of dietary protein while chymotrypsin activity decreased and also found some specific including unexplained effects of protein sources on trypsin or chymotrysin activity (D'Abramo *et al*., 1997).

2.3 Trypsin gene expression

Trypsin is a key enzyme for protein digestion in shrimp. Trypsin cleaves protein at specific peptide bonds (lysine, arginine, phenylalanine and tryptophan) in protein structure.

High specific activity of trypsin was related with salmon fed high quality protein and it also with high feed utilization but expression of related gene was not reported (Sunde *et al*., 2004). Mechanisms controlling trypsin gene expression in shrimp has not known, however, Noriega and Wells (1999) reported trypsin gene expression in mosquito, *Aedes aegypti*, which consist of 4 steps. First is synthesis of early trypsin gene which occurred without hormone stimulation. Step 2 after feed intake and digestion with existing enzyme until free amino acids are produced and resulted in stimulating of translation of early trypsin gene which triggers for protein digestion in step 3. After that the digestion products stimulate late trypsin gene. Trypsin gene expression in shrimp is related with molting which is a key process for crustacean growth. The highest trypsin gene expression was observed at early pre-molt stage (D_1) and sharply declined in late pre-molt stage (D_2-D_3) (Klein *et al*., 1996).

Moreover, starvation is also a factor affecting trypsin gene expression. In *L. vannamei*, Sanchez-Paz *et al*. (2003) reported the highest trypsin gene expression at 24 hours after starvation. In addition, nutritional factors affecting gene expression in *L. vannamei* were studied by Muhlia-Almazan *et al*. (2003). The results showed that shrimp fed 30% protein diets had greater trypsin gene expression than shrimp fed 15% and 50% protein diets but there is no evidence of effects of protein quality and protein substitution on trypsin gene expression.

2.4 Protein, amino acid, lipid and energy requirement in *L. vannamei*

Protein requirement in white shrimp is dependent on shrimp's size. Wyk (1999) reported that protein requirement of shrimp size range from 0.002-0.25, 0.25-1.0, 1.0-3.0 and >3.0 g was 50, 45, 40 and 35% protein, respectively. Kureshy and Davis (2002) reported the protein requirement for maximum growth in juvenile and adult *L. vannamei* utilizing three practical diets containing 16%, 32% and 48% dietary protein. On an isonitrogenous basis, the 32% protein diet produced significantly better weight gain compared to 16 and 48% protein diets. Broken line analysis was conducted on the growth responses for diets and each size of shrimp in order to determine the protein requirement for maximum growth. Protein requirement for maximum growth of juvenile shrimp was found to be 46.4 g DP/(kg body weight/day) when fed the 32% protein diet and 43.4 g DP/(kg body weight/day) when fed the 48% protein diet.

Subadult shrimp exhibited a maximum protein requirement of 23.5 g DP/(kg body weight/day) when fed a 32% protein diet and 20.5 g DP/(kg body weight/day) when fed a 48% protein diet. More important than dietary protein level, amino acid profile of dietary protein should meet requirement level in order to support good growth (Millamena *et al*., 2006). Akiyama *et al*. (1991) reported that essential amino acid for *L. vannamei* are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine at 5.8, 2.1, 3.5, 5.4, 5.3, 2.4, 4.0, 3.6, 0.8 and 4.0% of protein, respectively.

The lipid requirement of shrimp depends on their essential fatty acid profile and phospholipid content (Gonzalez-Felix *et al*., 2002). Polyunsaturated fatty acid was considered as an essential fatty acid for shrimp (Joseph and Meyer, 1975; Joseph and Williams, 1975), particularly linoleic acid (18:2n-6), linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Virtual University for Agricultural Trade, 2006). In general, terrestrial plant oils are high in 18:2n-6 and 18:3n-3 while the marine animal oils are high in 20:5n-3 and 22:6n-3. The optimum dietary level of highly unsaturated n-3 fatty acid, 20: 5n-3 and 22:6 n-3 for shrimp has been determined to range from 0.5-1% (Lovell, 1998) while the optimum level of the n-6 series of fatty acids is estimated to be approximately 0.5% (Lovell, 1998). Diets containing 0.5% n-6 and 0.5% n-3 fatty acids have provided for maximum growth of several shrimp species and total lipid level in diet should not exceed 10% (Lovell, 1998; Gonzalez-Felix and Perez-Velazquez, 2002). However, essential fatty acid requirement in different shrimp species are varied depending on species, culture conditions, interaction among essential fatty acids and total lipid level in diets. Gonzalez-Felix *et al*. (2007) evaluated the effect of various dietary lipid levels on quantitative requirement for essential fatty acids (EFA) by juvenile *L. vannamei* using three dietary lipid levels (3, 6 and 9%) and three dietary levels (0.5, 1 and 2%) of a mixture of n-3 highly unsaturated fatty acids (HUFA). Results showed that n-3 HUFA requirements in *L. vannamei* were met when supplied at 0.5% of diet, while in contrast, depressed growth was observed in shrimp fed diets with the HUFA mixture supplemented at 2%. Increasing lipid level (3-9%) affected lipid composition of shrimp by increasing lipid deposition in hepatopancreas and muscle tissue but without a significant effect on growth.

Gonzalez-Felix *et al*. (2003b) studied the nutritional value of dietary n-3 and n-6 polyunsaturated fatty acids for juvenile *L. vannamei* using diets containing 5% total lipid with a basal diet containing palmitic and stearic acid at 2.5% of diet. Five diets contained 0.5% dry weight of either linoleic, linolenic, arachidonic, eicosapentanoic (EPA) or docosahexaenoic acid (DHA). An additional diet evaluated combination of highly unsaturated fatty acids by supplementing at 0.5% of diet. The results showed that EPA and DHA had higher nutritional value than polyunsaturated fatty acid (linoleic, linolenic and arachidonic acid) and produced significantly ($p<0.05$) higher final weight, weight gain and total lipid in shrimp muscle.

Hu *et al*. (2008) studied ratio of lipid levels (5, 7.5 and 10%) and protein levels (30, 34, 38 and 42%) on growth of juvenile *L. vannamei*. The results showed that the diet containing 34% protein and 7.5% lipid with digestible protein/digestible energy of 21.1 mg/kJ was optimum and no protein-sparing effect was observed when dietary lipid level was increased.

Gonzalez-Felix *et al*. (2003a) evaluated nutritional value of dietary linoleic and linolenic acid for juvenile *L. vannamei* using a basal diet containing 5% total lipid supplied by 2.5% of palmitic and 2.5% of stearic acids. Six diets contained one of three levels (0.25, 0.5 and 1%) of either linoleic or linolenic acid and another three diets had different ratios of linolenic/linoleic (1, 3 and 9) at level of 0.5% of diet. An additional diet contained 0.5% of a mixture of n-3 highly unsaturated fatty acids. The results showed that highly unsaturated fatty acid of the n-3 family gave higher nutritional value than linoleic and linolenic resulting higher final weight and weight gain ($p<0.05$). Neither linoleic nor linolenic, alone or in combination, improved shrimp growth in comparison with those fed the basal diet.

Gonzalez-Felix (2009) evaluated the effect of three levels of docosahexaenoic acid meal (DHAM) and arachidonic acid meal (ARAM), produced by using a meal that had high levels of the desired fatty acid (0.23% DHAM-0.5% ARAM, 0.5% DHAM-0.1% ARAM and 0.75% DHAM-0.15% ARAM) and three n-3/n-6 dietary ratios (0.3, 0.8 and 1.8) on growth and survival of juvenile *L. vannamei* culture in low salinity. Two additional reference diets with menhaden fish oil or soy and flax oils (n-3/n-6 ratios of 1.8 and 1.7, respectively) were tested. The results showed no significant differences (at $p< 0.05$) and no significant interactions among treatments for final weight, weight gain or survival after 6-week feeding which confirmed that

supplement of DHA and ARA from alternative sources to fish oil is effective in promoting growth and survival.

Lipid requirement of broodstock was found to be similar with juvenile *L. vanamei*. Diets with total lipid at 8.1% gave the highest gonadosamatic index as compared to 8.8, 9.8 and 11.2% which total dietary lipid levels affected ovarian maturation in a negative way and total dietary levels of highly unsaturated fatty acid had no pronounced effect on ovarian maturation within rage 0.6-2.7% (Wouters *et al*., 2002)

Culture conditions have been reported to affect lipid requirement in shrimp. Zhu *et al*. (2009) reported a significant effect of salinity on growth of juvenile *L. vannamei* which was better at 30 ppt than those cultured at 2 ppt. At 30 ppt seawater condition 6% lipid diet gave a higher growth than those fed 8 and 10% lipid and at the same dietary lipid level shrimp fed 44% protein diet had significantly higher weight gain than those fed 38% protein diet. In contrast, at the 2 ppt seawater condition shrimp fed 8% lipid diets had only slightly higher growth than those fed 6 and 10% lipid when fed either 38 or 41% protein diets.

Hurtado *et al*. (2006) analyzed the effect of HUFA supplementation (3% vs. 34%) on juvenile *L vannamei* reared for 21 days at low (5 ppt), medium (30 ppt) and high salinities (50 ppt). The results showed that shrimp grown at 5 ppt had lower survival compared with control (30 ppt) or shrimp grown at 50 ppt but no significant effect on survival as a result of HUFA enrichment. In contrast, growth was significantly lower for shrimp grown at 50 ppt but this effect was compensated by the HUFA-enriched diet. The results demonstrated that growth at high salinities is enhanced with diets containing high HUFA levels but that diets have no effect on shrimp reared at low salinities.

In addition, lipid requirement of shrimp reared in green water with plenty of floc was less than in clear water as a results of absorption of different fatty acids such as 16:1n-7, 17:1, 20:4n-6, 20:3n-3 and 22:5n-6 from floc lipid. The nutritional contribution of the floc to shrimp in mesocosm culture eliminated the dietary source of fish oil and illustrates the importance of DHA and ARA to enhance shrimp survival in clear water conditions (Izquierdo *et al*., 2006).

Energy requirement in *L. vannamei* was not elucidated but Hajra *et al*. (1988) studied in *P. monodon* juvenile and found that at constant dietary protein level of 46% weight gain, feed efficiency and protein utilization increased with increasing dietary energy level up to 412.60 kcal/100 g (P/E = 112.2). Further, elevation in dietary energy content had no beneficial effect. Protein efficiency ratio (PER) remained negatively correlated to E/P ratio up to the optimum dietary energy level (412.60 kcal/100 g).

2.5 Protein sources and fishmeal replacement

Protein sources

Protein sources used as feed ingredients for aquatic animals include fishmeal, squid meal, shrimp shell meal, shrimp head meal and soybean meal. Fishmeal is a protein source with good amino acid balance and essential fatty acids meeting requirements for growth in aquatic animals. Squid meal, shrimp shell meal and shrimp head meal play a role as protein source and also act as feed attractants in shrimp diets. However, inclusion level in diet is less than fishmeal due to its imbalanced amino acid profile (Hertrampf and Piedad-Pascaual, 2000). Soybean meal is a cheaper protein source than fishmeal. Defatted soybean meal with denatured trypsin inhibitor is a soybean meal products act as feed ingredients because of its reasonable price for feed industry than full fat. The high digestibility and protein content promote the adoption of soybean meal by industry but the first limiting methionine is reduces its utility (Hertrampf and Piedad-Pascaual, 2000). Besides, protein sources from terrestrial animal processing by-product such as blood meal, hemoglobin meal, and hydrolyzed feather meal are being used as a replacer. However, levels of substitution are varied depending on protein sources in terms of nutritional quality and protein digestibility.

Hemoglobin meal is being used increasingly as an ingredient in the feed industry. Basically, hemoglobin meal is produced from uncoagulated fresh blood by centrifugal process at 4 $^{\circ}$C and obtained precipitate was spay-dried (Eurotec nutrition (Thailand), 2006). Hemoglobin meal is considered as a good quality protein source due to its good hygienic process during production which resulted in prevention of protein deterioration. Moreover, hemoglobin

meal contains high protein (approximately 92%) which is easily digestible with high lysine content, whereas methionine content is low (Hertrampf and Piedad-Pascaual, 2000). Excluding plasma portion of blood product influences protein digestibility. Australian snapper has higher ability to digest protein in hemoglobin meal (95.1%) than that of ring-dried blood meal (81%) (Booth *et al*., 2005).

Although there are varieties of protein ingredients, fishmeal is still a key protein sources for the good growth of animals. However, the demand of fishmeal is rising while production is declining which will lead to future shortage. Fishmeal price, as consequence is increasing which directly results in a higher feed cost. Thus, feed industry is attempting to seek other protein sources for fishmeal substitution.

Protein substituted for fishmeal

Results from many studies show the different of protein levels can be use due to many factors such as animals acceptance, imbalance of essential amino acid and digestible of that protein by animals which effect protein utilization of those ingredients. Many ingredients were investigated and have potential as replacer for fishmeal including blood meal (Dominy and Ako, 1988), soybean meal (Lim and Dominy, 1990; Mente *et al*., 2002), enzyme-hydrolyzed feather meal co-extrude with soybean meal (Mendoza *et al*., 2001), shrimp waste meal (Cruz-Suarez *et al*., 1993), meat and bone meal (Forster *et al*., 2003; Tan *et al*., 2005), barley grain and wheat gluten (Molina-Poved and Morales, 2004) and tuna visceral co-extruded with corn meal (Hernandez *et al*., 2004). Besides, protein supplement in form of floc particles which is bacterial and phytoplankton protein is also used in intensive shrimp pond.

In early research on protein substitution was perform with single protein sources and came up later with the combine one (Sudaryono *et al*., 1995; Bautista-Teruel *et al*., 2003; Forster *et al*., 2003; Suarez *et al*., 2009) . Single protein of plant protein, soybean meal, can be substituted for marine protein at 40% with no effect on growth whereas the substitution at 80% and 100% resulted in lowering feed intake due to unpalatable of the diets (Lim and Dominy, 1990) while Mente *et al*. (2002) reported that juvenile *L. vannamei* fed marine protein diets and diets substitute 50% marine protein with soybean meal gave not different growth. In contrast, terrestrial processing by-product blood meal which containing protein content has maximum

capability only 10% substitute without impaired growth (Tacon and Akiyama, 1997). Meat and bone meal is another protein source has a potential as a replacer which Tan *et al*. (2005) reported 60% substitution for fishmeal without impaired growth, survival rate, feed efficiency ratio, protein efficiency ratio and proximate composition of *L. vannamei* at the end of trial but using render meat and bone meal can be replace for fishmeal at 25% (Forster *et al*., 2003) which was showing the un-similarity even the same source of ingredient was also effected growth. Cruz-Suarez *et al*. (1993) revealed that using shrimp head meal as a replacer for fishmeal up to 18% gave the best growth compared with the lower one and also better than shrimp head including hulls meal at the same levels.

The combined protein substituted for fishmeal had study with partly aiming to employ the balancing of amino acid benefited from combination. Mendoza *et al*. (2001) studied fishmeal replacement with feather-enzymatic hydrolysates co-extruded with soya-bean meal in *L. vannamei*, the results showed increasing weight gain depend on hydrolysate-processing which enzymatic hydrolyzed feather meal gave greater weight gain than steam hydrolyzed feather meal and the combination of enzymatic hydrolyzed feather meal co-extruded with soya-bean meal at ration 2:1 can be substitute at 20% of diets without impaired growth which can be replaced fishmeal at 55%. Combination of barley-based fermented grains and wheat gluten at 1:1 was being study by Molina-Poveda and Morales (2004). The results showed maximum replacement at 66% was still containing amino acid balance and keep palatability of that diet. Co-extruded wet tuna viscera and corn meal is a combination that can replace fishmeal at 40% without impaired growth and can be improved diet palatability but unaffected feed conversion ratio (Hernandez *et al*., 2004).

Amino acid supplementation

Attempts to use cheaper protein sources comonally face with the limiting amino acid, in order to reduce depending on the expensive marine protein source through amino acid supplementation to achieve equivalent protein quality in term of chemical component was perform in animal feed including shrimp. Huai *et al*. (2009) studied dietary protein reduction with synthetic amino acids supplementation in juvenile *L. vannamei*, the results showed that commercially available synthetic amino acids supplementation of diets which 50% fishmeal was

substituted with soybean meal can improve growth but lower than single fishmeal diet. In addition, dietary crude protein containing 20% fishmeal could be reduced from 41.26 to 35.52% in the diets as long as synthetic amino acids were supplemented to the crude protein reduction.

Dominy and Ako (1988) compared 4 types of blood meal for fishmeal substitution in white shrimp diets; 1) ring-dried blood meal (RD), 2) acidulated and sun-dried blood meal (AS), 3) acidulated and sun-dried blood meal mixed with methionine crystal (ASAM) and 4) acidulated and sun-dried blood meal coupling with covalently linked methionine (ASCM). Results showed non-significant difference of weight gain, survival rate and FCR. However, AS and ASAM diets gave a lower production that the others. Results also indicated that supplementation of methionine in the form of covalently link methionine is effective for shrimp diet.

2.6 Protein quality

Protein quality is a measure of the usefulness of a dietary protein for growth and maintenance of tissue, and, in animals, production of meat and other products. The quality of individual proteins is unimportant in mixed diets because of complementation between different proteins. Two methods of measurement are used to estimate protein quality, biological assays and chemical analysis (Fennema, 1996).

Biological assay uses animals incorporate for evaluating which this method have many value such as biological value (BV) that is the proportion of absorbed protein retained in the body (i.e. taking no account of digestibility), Net Protein Utilization (NPU) or protein productive value (PPV) which is the proportion of dietary protein that is retained in the body under specified experimental conditions (i.e. it takes account of digestibility; NPU = BV × digestibility), Protein Efficiency Ratio (PER) which is the gain in weight of growing animals per gram of protein eaten. Chemical analysis showed chemical score of the tested protein which is amino acid profile.

The quality of any protein sources are not the same even though being the same protein source because it cause by various factor. For example, protein of different sources have different protein structure and containing varying amino acid profile which might be deficiency

in some amino acid, even such a definitely the same in chemical component, result of processing leading to the digestibility changing of that protein and also effect to the protein utilization efficiency that it might be better or get worse can be occur. Fishmeal is a good protein quality which had the highest PPV compared to soybean meal (Venou *et al*., 2006; Tantikitti *et al*., 2005), an adverse effect on PPV was observed by increasing soybean meal inclusion levels diet fed to gilthead seabream (Venou *et al*., 2006) and Asian seabass (Tantikitti *et al*., 2005). The similar results were observed in Atlantic salmon fed diet replaced fishmeal with whole mince herring fish silage (Espe *et al*., 1999). Different protein sources had different protein quality was observed in juvenile catfish fed diet (Fagbenro and Jauncey, 1995). Catfish fed diet which co-dried lactic acid fermented fish silage of whole tilapia with poultry by-product meal gave the highest PPV followed by co-dried with meat and bone meal, hydrolyzed feather meal and soybean meal. Ash content in diet was not affected PER and PPV in Atlantic salmon (Toppe *et al*., 2006). Increasing ash content was due to increasing fishmeal replacement with fish bone from 15% to 45%. In addition, protein quality was affected by protein level in diets. Abdel-Tawwab *et al*. (2009) reported that Nile tilapia fed diets containing 25% protein gave the highest PER and PPV whereas that fed 45% protein gave the lowest PER and PPV compared to 25 and 35% protein diets which the decreasing protein quality which was suggesting that the excess dietary protein might be deaminated and produced ammonia rather than aggregated to muscle growth. Protein quality affected by the diet component was found in juvenile southern rock lobster (Ward *et al*., 2003). Lobster fed isoenergetic diets containing whole fish silage ranged from 25 to 45% protein at 5% and 9% lipid gave decreasing PER and PPV with silage inclusion level increment but the highest PPV peak of 5% lipid diet was at 29% protein whereas that of 9% lipid diet was found at 36% protein.

2.7 Protein digestibility and evaluation

Digestibility is a key factor affecting protein utilization of different protein ingredients. Protein digestibility of ingredient and pelleted diets can be assessed through *in-vitro* and *in-vivo* digestibility. *In-vitro* protein digestibility can be investigated using exact amount of crude enzyme reacting on known amount of protein substrate, then measuring obtaining amino

acid products. This rapid method is proper for evaluating ingredients or diets prior to use. The *in-vitro* protein digestibility using crude enzyme has limitation due to specie different specific activity of crude enzyme influence by diets (Divakaran *et al*., 2004).

In-vivo protein digestibility is evaluation conducted on animal which gives a true evaluation of protein utilization demonstrated in growth responses and feed utilization in the same trial. However, *in-vivo* digestibility is time consuming and expensive.

Like other nutrients, protein digestibility coefficient can be classified into two forms as follows.

1. True digestibility which is considering nitrogen excretion accompany with feces such as enzyme, peptide and mucus.

2. Apparent digestibility which nitrogen excretion in feces is exclude because it is difficult to distinguish the metabolic losses due to these other components from unabsorbed materials originating from the feed.

Apparent digestibility is practically employed for nutrient digestibility studies and apparent protein digestibility can be calculated as follows.

$$\text{Protein digestibility (\%)} = 100\text{-}100\left[\frac{\text{\% marker in diet}}{\text{\% marker in feces}} * \frac{\text{\% protein in feces}}{\text{\% protein in diet}}\right]$$

Factors affecting protein digestibility

Nutrient digestibility depends on many factors such as species and age of animals, cultured conditions, nutrient structure, anti-nutritional factors and nutrient interactions. Nutritional factors are an important due to theirs influenced on feed price. Besides, using nutrient with efficiency carelessly can be deteriorated aquaculture media.

Lee and Lawrence (1997) reported that animal protein had high protein digestibility than plant protein but mixed protein had moderate protein digestibility. For example, shrimp meal and fishmeal had protein digestibility ranged from 80-95% and mixed protein had protein digestibility of 40-70%. Besides, protein digestibility also mainly related with protein content in diet than effect from processing.

Fox *et al*. (1995) reported form of amino acid supplement effected protein digestibility. Wheat gluten supplemented with crystal amino acid showed higher protein digestibility than non supplement and lysine covalent link one.

Catacutan (1991) reported that levels of carbohydrate in diets influence dry matter digestibility but not protein digestibility in *P. monodon*. In contrast, Shen and Lui (1992;1993) cited by Lee and Lawrence (1997) reported the relation of levels of carbohydrate on protein digestibility in *P. orientalis*.

Lee and Lawrence (1997) reported that using alginate at 3% in diets was not effect protein digestibility but it resulted in depletion of amino acid lysine and valine at 4%

Bendiksen *et al*. (2003) reported that significant protein digestibility of Atlantic salmon (*Salmo salar* L) increased with substitute fish oil with vegetable oil.

Protein quality and protein digestibility

Protein digestibility of many protein ingredients are varying due to physical property of them. For example, *L. vannamei* had ability to digest and absorb casein at 99% meanwhile it is being only 75% in shrimp head meal (Akiyama *et al*., 1989 cited by Lee and Lawrence, 1997). Demonstrating variation is result from easy digestible protein structure of casein than shrimp head meal enhancing feed utilization and leading to good growth. However, Interaction with other ingredients of protein is also affected protein digestibility particularly plant protein which normally containing phytate. Phytate or phytic acid consist of glucose like structure called mio-innosital which contact with phosphate group (PO_4^{2-}) thus it can interact with cat ion and also protein. In case of protein complex interaction compound it is directly adverse effect protein digestibility. Storebakken *et al*. (1998) and Baruah *et al*. (2004) reported that digesting soy protein concentrate with phytase resulted in increasing protein digestibility and increasing protein gain in Atlantic salmon.

Many researchers reported protein quality changed by processing and affect growth both impair and enhance growth depend on the aim of processing output but a few of them reported the relation of processing and protein digestibility. Sunde *et al*. (2004) reported that low quality protein with high disulfide groups resulted from heating processing gave non-

significant specific growth rate but gave lower feed utilization than high quality protein. Besides, researchers were also reported that greater feed utilization related to the higher specific activity of trypsin and chymotrypsin.

2.8 Lipid sources and protein-sparing effect

Lipids must be added to the diet in order to meet the requirements of fish or shrimp for certain essential fatty acids and sources of energy for metabolism. Many different lipids are used in formulated feeds, including lipids of both plant and animal origins from both terrestrial and aquatic environments. Terrestrial lipids of animal origin, such as pork fat and beef tallow contain high levels of saturated fatty acids and are poor sources of essential fatty acid of n-3 and n-6 series. Lipids produced from the seeds of a number of plant species, such as soya and sunflower contain high levels of unsaturated fatty acids of the n-6 series, but the best sources of the essential n-3 series fatty acids are lipids of marine origin (Jobling, 1994). Regost (2003) reported a slight decrease in growth of turbot (*Psetta maxima*) fed total fish oil replacement with vegetable oil diet. Similar results were also obtained in red hybrid tilapia (*Oreochromis* sp.) (Bahurmiz and Ng, 2007) and juvenile black seabream (*Acanthopagrus schlegeli*) (Peng *et al*., 2008). However, uncertainty of fish oil quantity and price leading to a search for alternative oil source.

Tan *et al*. (2009) reported lipid quality on growth performance, hepatic fatty acid profiles and intermediary metabolism of juvenile yellow catfish *Pelteobagrus fulvidraco*. Results showed that dietary linolenic acid to linoleic acid ratios significantly influenced viscerosomatic index and hepatosomatic index ($p < 0.05$), but not condition factor ($p > 0.05$). Body composition was also significantly influenced by dietary linoleic acid to linoleic acid ratios ($p < 0.05$). Generally, liver fatty acid compositions reflected dietary fatty acid profiles. Declining linoleic acid and increasing linolenic contents in liver were observed with the increasing dietary linolenic acid/linoleic acid ratios ($p < 0.05$). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) increased with the increasing linolenic acid to linoleic acid ratios, suggesting that yellow catfish could elongate and desaturate C18 polyunsaturated fatty acids into highly unsaturated fatty acids . As a consequence, the n–6 fatty acids (FA) declined, and total n–3 FA

and n–3/n–6 ratios increased with the dietary ratios of linolenic acid/linoleic acid ($p < 0.05$). Dietary linolenic to linoleic ratios significantly influenced several enzymatic activities involved in liver intermediary metabolism ($p < 0.05$), such as lipoprotein lipase, hepatic lipase, pyruvate kinase, succinate dehydrogenase, malic dehydrogenase and lactate dehydrogenase, suggesting that dietary linolenic acid/linoleic acid ratios had significant effects on nutrient metabolism in the liver.

Protein sparing effect

Lipid, a key energy source, plays an important role in sparing protein in different species of aquatic animals such as salmon, rainbow trout and tilapia (Beamish and Thomas, 1984; De Silva *et al*., 1991; Azevedo *et al*., 2002). The ability to utilize lipid rather than protein as energy source can lead to a decreased loss of ingested protein by catabolism (Refstie *et al*., 2001; Williams *et al*., 2003) which is clearly observed when the amount of dietary protein consumed is low (De Silva *et al*., 1991). The protein sparing effects by dietary lipid levels have been reported in different fish species and varied between species (Lie *et al*., 1988; De Silva *et al*., 1991; Alam *et al*., 2009). In rainbow trout, reduced excretion of nitrogen, a measure of amino acid metabolism, was observed when a diet containing high amount of lipid (20%) at 0.5% of body weight per day, but such sparing effect was not evident when they were at 0.1% of body weight per day (Beamish and Thomas, 1984). De Silva (1991) also found an improved net utilization in red tilapia fed to satiety with diets containing increasing amount of lipid to 18% of diet, but increasing dietary lipid beyond 18% of diet caused a reduction in net protein utilization.

In shrimp, effects of protein sparing actions of energy nutrients have been contradictory. Capuzzo and Lancaster (1979) reported that decreasing protein levels in formulated feeds led to increasing dependency on dietary carbohydrate as an energy source in post-larval American lobsters and protein efficiency ratios were inversely correlated with the protein levels of diets. In *P. Japonicus*, growth and survival of the prawn fed different levels of dietary proteins were varied with dietary carbohydrate levels but not with dietary lipid levels (Teshima and Kanazawa, 1984). Hu *et al*. (2008), on the other hand, reported that *L. vannamei* fed the 75 g of lipid/kg of diet had only slightly higher growth than that fed 50 g of lipid/kg of diet at the same dietary protein level and even a little decline in growth with the further increase

of dietary lipid to 100 g/kg of diet. Shrimp fed the diet with 420 g/kg protein and 75 g/kg lipid had the highest specific growth rate. However, shrimp fed the diet with 340 g/kg protein and 75 g/kg lipid showed comparable growth and had the highest protein efficiency ratio, energy retention and feed efficiency ratio among dietary treatments. Triglycerides and total cholesterol in the serum of shrimp increased with increasing dietary lipid level at the same dietary protein level. Body lipid and energy increased with increasing dietary lipid level irrespective of dietary protein. Results of this study showed that the diet containing 340 g/kg protein and 75g/kg lipid with digestible protein/digestible energy of 21.1 mg/kJ is optimum for *L. vannamei* and the increase of dietary lipid level exerted no effect on sparing protein.

CHAPTER 3

Effects of Fishmeal Quality on Growth Performance, Protein Digestibility and Trypsin Gene Expression in Pacific White Shrimp (*Litopenaeus vannamei*)

3.1 Abstract

A seven week feeding trial was conducted using five diets to examine effects of fishmeal (FM) quality on growth performance, apparent crude protein digestibility and expression of gene regulating trypsin in *Litopenaeus vannamei*. Each test diet was fed four times daily to four groups of shrimp with an average initial weight of 2.2 g/shrimp. The shrimp fed the diet with premium grade FM S1 had the highest final weight, weight gain, specific growth rate with the best feed conversion ratio (FCR) and protein utilization efficiency. Shrimp fed diets containing premium grade S2, grade 1 FM and grade 2 FM showed similar growth performance second to that of the premium grade S1. Growth performance and feed utilization efficiency of imported FM (Chile) fed group was the lowest. FCR of shrimp fed grade 2 FM and imported FM was significantly the highest ($p<0.05$).

In-vitro protein digestibility using crude enzyme extract from shrimp fed reciprocal diet was not significantly different among treatments but *in-vivo* protein digestibility of premium grade FM S1 fed group was the highest (92.06%) correlated with the highest trypsin gene expression.

3.2 Introduction

L. vannamei has become one of the most productive species which mostly cultured under super intensive and high cost culture system. Success in this culture is highly dependent on the availability of well balanced, pathogen control, nutritionally complete and cost effective formulated feeds (Bautista-Teruel *et al*., 2003; Cuzon *et al*., 2004). Among macro nutrients (protein, lipid and carbohydrate), protein is a key nutrient for muscle growth and a key protein source is fishmeal due to the amino acid balance with high levels of essential amino acids.

In Thailand, fishmeal is classified into premium grade, grade 1, grade 2 and grade 3 based on protein, fat and ash contents. The fat content ranges from 4 to 20%, and the ash content is highly variable, ranging from about 11-12% in anchovy meal to over 23% in whitefish meals made from filleting waste. However, those criteria scarcely imply the growth performance at all.

Fishmeal quality can be used to predict animal growth as indicated by the good growth of gilthead seabream fed good quality fishmeal (Caballero *et al*., 1999) whereas fishmeal grade cannot. Fishmeal of the same protein level may differ in essential amino acid composition, essential amino acid/non essential amino acid ratio, and structure of protein which directly affect protein digestibility resulting in feed utilization and ultimately affecting growth. Generally, fishmeal is rich in lysine which is low in many plant protein such as corn (Fernandez *et al*., 1994) However, fishmeals made entirely from offal (fish frames) have an amino acid content that is typically 10% lower than that of meal made from the whole fish (D'Abramo *et al*., 1997). Fishmeal obtained from slightly decomposed raw materials gave the highest growth has been described in crustacean nutrition by D'Abramo *et al*. (1997). In addition, fishmeal processed at high temperature especially more than 90 $^{\circ}$C gave low weight gain (Pike *et al*., 1990).

Protein digestibility is an important indicator of protein utilization assessment of any protein source. Protein digestibility of fishmeal derived from different sources differ such as the study reported by Kangsen (1986). The results showed that Peruvian fishmeal (88%) gave higher protein digestibility than Chinese fishmeal (71%) when incorporated in the diet for *P. orientalis*. In contrast, similarity of protein digestibility was also found for example study conducted by Forster and Gabbott (1971) the result showed that *Palaemon serratus* could utilize Norwegian fishmeal, herring, at 89% whereas 87% of that Peruvian fishmeal. Smith *et al*. (1985) found a positive correlation between protein digestibility and growth rate in *L. vannamei*.

Trypsin and chymotrypsin encoding gene expression involved digestive process which is controlled by hormonal and central nervous system. Muhlia-Almazan *et al*. (2003) reported that the trypsin and chymotrypsin encoding genes can be induced by dietary protein levels in *P. monodon*.

Aside from digestive protease enzyme encoding gene expression in shrimp, trypsin genes in *Anopheles gambiae* have been induced by blood meal (Muller *et al*., 1995) as

well as late trypsin gene expression in *Aedes aegypti* which is dietary control. In addition, the transcription of the late trypsin gene in the midgut of the mosquito, *Aedes aegypti*, is activated by early trypsin activity which is a part of transduction system (Barillas-Mury *et al*., 1995).

This study was carried out to test the hypothesis that good fishmeal quality with a balanced amino acid profile gives a higher growth performance, *in-vitro* and *in-vivo* protein digestibility and trypsin encoding gene expression in *L. vannamei*.

3.3 Materials and Methods

3.3.1 Experimental diets and leaching tests

Five diets were formulated using different fishmeals, imported FM (Chile), premium grade FM (S1) produced from sardine, premium grade FM (S2) produced from round scad and sardine, grade 1 FM produced from mixed FM and grade 2 FM produced from surimi processing by-product. The diets contained protein and lipid at 42% and 8% of diet, respectively.

The coarse ingredients were finely ground to pass through a 30 mesh screen. Dry ingredients were mixed using Hobart mixer (A200T ML 104568, Troy, Ohio, USA) for 10 min, then lecithin and oil were gradually added and mixed for a further 5 min. Distilled water was gradually added at 35% of diet and mixed for another 10 min. The resulting mash was pelleted using pelleted mill with a 2 mm diameter pore size die and cut into 2 mm length pelletes. Pelleted diets were dried at 60 $^{\circ}$C for 24 h. The dried diets were sieved through a 2 mm diameter mesh screen and stored in polyethylene bags at -20 $^{\circ}$C in the dark until used. The proximate composition of ingredient and experimental diets was determined (AOAC, 1995). The amino acid profile of diets was determined by HPLC (AOAC, 1995).

Diet leaching test was performed using three replicates according to the method modified from Aquacop (1978) and Cruz-Suarez *et al*. (2001). Five g of the pellets were put on fine mesh baskets and immersed in water for 1 h with aeration simulating cultured conditions in glass aquaria. The percent dry matter loss (%DML) was calculated as:

Table 1 Composition (g/100g), proximate composition (% as fed basis) and leaching loss (dry matter basis) of experimental diets

Ingredients	Experimental diets				
	Imported FM (Chile) (65.86% protein)	**Premium grade FM (S1) (65.25% protein)**	**Premium grade FM (S2) (71.11% protein)**	**Grade 1 FM (64.91% protein)**	**Grade 2 FM (55.22% protein)**
Fishmeal	43.00	43.50	39.50	44.00	52.5
Squid meal	8.00	8.00	8.00	8.00	8.00
Wheat flour	20.00	20.00	20.00	20.00	20.00
Rice flour	12.29	11.19	14.69	10.99	3.39
Wheat gluten	6.00	6.00	6.00	6.00	6.00
Lecithin	2.00	2.00	2.00	2.00	2.00
Tuna fish oil	0.60	1.20	1.70	0.90	0.00
Vitamin mix[1]	0.33	0.33	0.33	0.33	0.33
Mineral mix[2]	4.00	4.00	4.00	4.00	4.00
Vitamin C	0.10	0.10	0.10	0.10	0.10
Zeolite	1.50	1.50	1.50	1.50	1.50
BHT	0.02	0.02	0.02	0.02	0.02
Cholesterol	0.50	0.50	0.50	0.50	0.50
Vitamin E	0.15	0.15	0.15	0.15	0.15
CMC	1.00	1.00	1.00	1.00	1.00
Cr_2O_3	0.51	0.51	0.51	0.51	0.51
Proximate composition (% as fed basis) and leaching loss (dry matter basis) of experimental diets					
Protein	43.41	42.06	41.96	42.99	43.49
Crude fat	11.26	10.41	10.23	11.30	11.14
Ash	7.03	9.55	7.25	12.36	16.99
Leaching loss (%)	11.44	11.61	10.28	11.12	8.51

[1] vitamin mix (in 1 kg of vitamin mix): retinol, 3500,000 IU; cholecalciferol, 800,000 IU; tocopherol, 40g; menaquinone, 15g; thiamine, 20g; riboflavin, 15g, pyridoxin, 20g; cyanocobalamine, 10mg; niacin 40g; panthothenic acid, 40g; folic acid, 4g; biotin, 400 mg; inositol, 150g.

[2] mineral mix (g/kg mineral): K_2HPO_4, 40; $Ca_3(PO_4)2$, 5.5; $MgSO_4$7H2O, 6.1; $NaH_2PO_4$2H_2O 16; Cellulose 828.

%DML = 100*(DWd-DWwid)/DWd; where DWd and DWwid are the dry matter weights of the diet before and after immersion, respectively.

3.3.2 Growth trial

Shrimp, culture and feeding

Juvenile *L. vannamei* shrimp were obtained from Somchai Farm, Satun province, Thailand. The shrimp were stocked and acclimatized in a cement tank for 15 days and fed a commercial feed which contain 40% protein and 4% lipid. Twenty-five shrimp with an individual initial weight of 2.29±0.01 g were selected and randomly distributed into each of 20 glass aquaria (45*45*115 cm) containing 200 L of natural seawater (water flow rate 33.26 L/h, water temperature 26-30 °C, salinity 29-33 ppt). Five treatments were randomly assigned to four replicated aquaria and fed respective diet. Feeding was done by hand to satiation determined by slow or no response to the diet, 4 times a day at 8.00 am, 12.00 am, 5.00 pm and 10.00 pm for 7 weeks. Uneaten feed was collected for feed intake correction.

Sampling

At the end of the feeding period, six shrimp from each aquarium were sampled for proximate analysis. Two shrimp were decapitated and the hepatopancreas were fixed in TRIzol reagent and kept at -80 °C until analysis. Another two shrimp were weighed, decapitated and hepatopancreas were taken then pooled from each replicated aquarium for *in-vitro* protein digestibility determination.

3.3.3 *In-vitro* protein digestibility

Enzyme extraction and activity determination

In-vitro protein digestibility of the experimental diets was determined using crude enzyme extract from the hepatopancreas from the shrimp in the experiments described above. The crude enzyme extract was prepared and the *in-vitro* protein digestibility study was performed using the method modified from Bassompierre (1997). Crude enzymes were extracted from the hepatopancreas and homogenized (1:10 w/v) in 0.05 M Tris buffer pH 7.5 at 4 °C. The

homogenate was centrifuged twice at 12,000 * *g* for 30 min at 4 °C. The crude enzyme was obtained and kept at -80 °C for further analysis use. Protein in extracted enzyme was measured by a modified Lowry's Method using bovine serum albumin (BSA) as a standard. Crude enzyme was diluted to 1 mg protein/mL before *in-vitro* digestion study. The trypsin activity of the crude enzyme extract was determined using BAPNA as a substrate by mixing 950 µL of 0.1 M BAPNA and 50 µL of crude enzyme, incubated at 37 °C for 10 min, then reaction was terminated by adding 100 µL of 30% Trichloroacetic acid and measured for an absorbance at 410 nm. The enzyme activity was calculated as follows (Rathore *et al*., 2005):

Unit of Enzyme activity (µmole/mL/mg protein)

$$= \frac{\text{(Abs at 410 nm/min) * 1000 mL * mL of reaction volume}}{\text{Extinction of chromagen * mg protein in reaction mixture}}$$

The molar extinction coefficient of *p*-nitroanilide is 8800.

Preparation of feed for enzyme digestibility assay

A sample of each diet was ground and weighed to the exact weight at 30 mg protein calculated using dietary protein content. Forty mL of 0.01 M phosphate buffer (pH 7.8) and 1 mL of 0.5% chloramphenical (in 96% ethanol) were added and mixed thoroughly. The mixture was incubated at 30 °C for 18 h in a shaking water bath.

Pre-digestion concentration

Prior to sample incubation, 0.5 mL of mixture from each treatment was sampled as a control, immediately heated at 100 °C for 5 min to terminate the enzyme activity, rapidly frozen at -80 °C for later determination of total reactive amino group using the trinitrobenzene sulfonic acid (TNBS) assay as described below.

Post-digestion concentration

Digestion concentration was determined by adding 0.5 mL of the crude enzyme extract (1 mg/mL of protein). The digestion process was performed in a shaking water bath for 18 h at 30 °C. At the end of incubation time, 1 mL of each digested mixture was sampled, immediately heated at 100 °C for 5 min and rapidly frozen at -80 °C for the later determination of free reactive amino group of the peptides using the trinitrobenzene sulfonic acid (TNBS) assay as described below.

Determination of free reactive amino acid groups

Dilution of 0.2 mL of either the undigested control or the digested mixture with 2 mL of 0.05 M phosphate buffer pH 8.2 were mixed thoroughly with 1 mL of 0.1% TNBS in 0.01 M phosphate buffer and were incubated at 60 °C for 1 h in the dark. The reaction was stopped by adding 1 mL of 1 N HCl and cooling to room temperature. The absorbance was measured at 420 nm and the concentration of free amino group was calculated using DL-alanine as standard. *In-vitro* digestibility was expressed as mole alanine equivalent liberated reactive amino group cleaved peptides per 200 µL sample.

Alanine equivalent liberated (mole) = alanine conc. (g/L) * (1/89.10 g/mole)* (0.2 mL/L)

3.3.4 *In-vivo* protein digestibility

Apparent digestibility coefficients (ADC) of dietary crude protein in diets were measured. Diets were prepared as described above with reducing chromic oxide (Cr_2O_3) as a marker which was included at 0.5% of diet.

After growth trial termination, feeding was continued with diets containing chromic oxide for 30 days. Feces collection commenced 2 days after changing to chromic oxide diets by siphoning method twice a day at 2.00 pm and 8.00 pm. Feces were separated from feed particles kept at -20 °C and oven dried at 105 °C. Determination of chromic oxide was carried out according to the method of Lall (1991). The ADC was estimated using the following equation.

$$\text{ADC} = \%\ \text{of protein digestibility} = 100 - \left(100 * \frac{I_a}{I_h} * \frac{P_h}{P_a}\right)$$

ADC = apparent digestibility coefficient

Where I_a = % Cr_2O_3 in feeds; I_h = % Cr_2O_3 in feces; P_a = % crude protein in food; P_h = % crude protein in feces.

3.3.5 Trypsin gene expression

Trypsin gene expression was studied using 2 steps RT-PCR. First step cDNA synthesis, total RNA was extracted from the hepatopancreas of shrimp fed six different diets using TRIzol reagent. Intact total RNA were used for reverse transcription; cDNA were synthesized from each individual sample using the Superscript III™ first-strand synthesis system for RT-PCR. Reverse transcription were performed using 8 μL (100 ng/μL) total RNA, 1 μL of (10 mM) dNTP mix and 1 μL of (50 ng/μL) random hexamer. The reaction mixtures were incubated for 5 min at 65 °C then further for 2 min at 4 °C. After 2 min at 4 °C of incubation, the reactions were added 10 μL of cDNA synthesis mixture which containing 2 μL (10X) RT buffer, 4 μL of (25 mM) $MgCl_2$, 2 μL of (0.1 M) DTT, 1 μL of RNase out and 1 μL of (50 units) Superscript III™ RT. The total volume of combined two portion mixture was 20 μL. Then the reaction extend incubated for 10 min at 25 °C, 50 min at 50 °C and 5 min at 85 °C after that added 1 μL of RNase inhibitor and extend incubated for 20 min at 37 °C which the reaction ended up and hold at 4 °C until used for second step.

Second step RT-PCR, trypsin primers for PCR amplification were based on three trypsin genes reported for *L. vannamei* (Klein *et al*., 1996). Primer sequences were Forward trypsin- CTCAACAAGATCGTCGGAGGAACTGA- and Reward trypsin - GACACTCGTCGTCAGAACACGATG- that matched positions 81-106 and 545-567, respectively.

PCR amplifications were performed in a 25 μL final reagent mixture containing 12.5 μL of H_2O, 2.5 μL of (10X) PCR buffer, 1.5 μL of (25 mM) $MgCl_2$, 1 μL of (10 mM) dNTP mix, 1 μL (6 μM) of each primer, 5 μL of the obtained cDNA (100 ng/μL) of each sample and 0.5 μL of Tag DNA polymerase. A thermocycler was used with the following program : 5 min at 95 °C, 1 min at 94 °C, 1 min at 54 °C and 1 min at 72 °C (35 cycles); and over-extension step for 10 min at 72 °C. The resulting PCR products for trypsin were analyzed in a single 1.5% agarose gel and stained with ethidium bromide (Sambrook and Russell, 2001)

The intensity of the bands in the obtained gel images were evaluated relative to that of EF-1 alpha (Wongpanya *et al*., 2007) using Scion Images program for window version 4 (Phongdara *et al*., 2006).

3.3.6 Statistical Analysis

Growth performance, survival rate, feed utilization efficiency, protein digestibility and trypsin gene expression data were analyzed using an analysis of variance to determine if significant differences exist among treatment means. The Tukey's HSD test was used to determine significant differences between treatments. A 5% error rate for significance was used for analyses.

3.4 Results

3.4.1 Amino acid and proximate composition of experimental diets

Glutamic acid was a major amino acid in all five diets with more than 12% of total protein. There was a similar amino acid profile of five diets although premium grade FM (S2) diet was highest level whereas grade 1 FM diet was the lowest in same amino acid comparison. Total essential amino acid content in diets varied regardless of fishmeal grade but grade 2 FM diet was the lowest. EAA/NEAA of imported FM (Chile), premium grade FM (S1), premium grade FM (S2), grade 1 FM and grade 2 FM were 0.68, 0.73, 0.69, 0.73 and 0.65, respectively (Table 2).

Proximate composition of diets was similar except ash content and leaching loss (Table 1). Grade 1 FM and grade 2 FM contained higher ash content than others which was 12.36 and 16.99%, respectively.

3.4.2 Survival rate

Shrimp fed premium grade FM (S1) diet had the highest survival rate (88%) while the others ranged from 78 to 86% (Table 3) without significant difference ($p>0.05$).

3.4.3 Growth and Feed utilization

Growth performance (Table 3) can be divided into three groups; the highest growth (Premium grade FM (S1) fed shrimp), medium growth (premium grade FM (S2), grade 1 FM and grade 2 FM diet fed shrimp), and significantly ($p<0.05$) the lowest growth (imported FM (Chile) fed shrimp). Shrimp fed premium grade FM (S1) diet had the highest final weight (10.28 g/shrimp), weight gain (7.99 g/shrimp) and specific growth rate (2.98) followed by grade 1 FM, premium grade (S2) and grade 2 FM fed shrimp respectively. Significantly the lowest growth was imported FM (Chile) diet fed shrimp having 8.15 g/shrimp of final weight.

Feed intake (Table 4) of imported FM (Chile) diet fed shrimp was significantly ($p<0.05$) the lowest at 8.79 g/shrimp, whereas that of others ranged from 10.10 to 10.33 g/shrimp and significantly ($p<0.05$) the highest at 11.06 g/shrimp was grade 2 FM diet fed shrimp. The feed conversion ratio (FCR) of shrimp fed different diets gave varying score regardless of feed intake (Table 4). Grade 2 FM diet gave the highest FCR with 1.55 g/shrimp followed by imported FM (Chile), premium grade (S2), grade 1 FM and premium grade FM (S1) diet giving significantly ($p<0.05$) the lowest (1.28 g/shrimp). Protein efficiency ratio (PER) and protein productive value (PPV) of respective diets (Table 4) gave a similar trend which premium grade FM (S1) diet showed significantly the highest followed by grade 1 FM, premium grade FM (S2), imported FM (Chile) and grade 2 FM diet showing the significantly the lowest.

The proximate composition of shrimp at the end of the trial (Table 5) was not different among treatments ($p>0.05$).

3.4.4 Protein digestibility

In-vitro and *in-vivo* protein digestibility (Table 6) of the diets in this study were similar. *In-vitro* digestibility in this study was performed using both commercial diet induced enzyme and reciprocal diet induced enzyme. The obtained *in-vitro* digestibility showed different results between the two methods which commercial diet induced method gave the fluctuation of results among treatments regardless of tested diets whereas experimental induced method gave the less fluctuation and showed a better data due to responsible for the tested diets.

In-vitro digestibility using experimental diet induced enzyme was not significant different among treatments whereas *in-vivo* digestibility of premium grade FM (S1) diet gave the highest followed by imported FM (Chile) and premium grade FM (S2) without significant different. Grade 1 FM diet gave significantly lower than above group and grade 2 FM gave significantly the lowest *in-vivo* digestibility. In addition, shrimp fed grade 1 FM and grade 2 FM secreted greater amounts of feces, particularly, the highest feces of grade 2 FM diet fed shrimp (Table 6).

3.4.5 Gene expression

Trypsin gene expression is shown in Figure 3. Shrimp fed premium grade FM (S1) diet showed the highest gene expression whereas shrimp fed imported FM (Chile) or premium grade FM (S2) or grade 1 FM showed lower score and shrimp fed grade 2 FM showed unexpression of gene.

3.5 Discussion

Diets produced from different FM had similar amino acid profile and proximate composition except ash content. The highest ash content in the grade 2 FM diet was due to high bone content in the raw materials. Similarly, high ash content in grade 1 FM could be from mixed protein sources. Imported Chilean fishmeal normally produced from single species with similar process but might have a longer storage time.

Shrimp fed the premium grade FM (S1) diet had the highest growth (final weight, weight gain and SGR) with the best FCR, PER and PPV. The best growth performance of this treatment was due to good amino acid balance and high EAA/NEAA of the diet. Shrimp fed this diet also had good feed intake and protein digestibility which provided the amino acids for muscle growth. Premium grade (S2) diet had similar feed intake and also had good protein digestibility but it had lower amino acid balance, particularly EAA/NEAA which was 0.69 leading to compromised growth compared to premium grade FM (S1) diet. Grade 1 FM diet had higher feed intake than previous diets but its lower protein digestibility was a cause of lowering the benefit of good amino acid balance with 0.73 of EAA/NEAA. The good growth of grade 2 FM diet fed shrimp caused by the higher feed intake. The less protein digestibility leading to the low ability to utilize the benefit of more amounts feed intake so that it affected growth regardless of its good amino acid balance with 0.73 of EAA/NEAA.

Significantly, the lowest growth of imported FM (Chile) diet fed shrimp caused by mainly on feed intake together with a slightly low protein digestibility and lower ratio of EAA/NEAA of 0.65. This diet was produced using grade 1 fishmeal from pelagic fish and imported from Chile. The length of time for transportation of this fishmeal necessitated high inclusions of antioxidant to prevent lipid oxidation which may affect feed acceptance and feed intake (Laohabanchong *et al*., 2009).

Growth responses caused by amino acid imbalance have been reported in many studies. Mengqing and Aksnes (2001) reported that shrimp (*Penaeus chinensis*) and red seabream fed the feed with good quality fishmeal showed significantly better feed conversion ratio, weight gain and protein digestibility than low quality fishmeal and Peru fishmeal. Similarly, Atlantic salmon fed with the diet containing amino acid imbalance due to the high temperature processing gave the lower growth than control because of high disulfide group forming affected the utilization efficiency of the diet (Sunde *et al*., 2004).

Growth depression due to amino acid imbalance effects of protein sources was also found in *L. vannamei* (Mente *et al*., 2002). The tested diets were a mixture of fishmeal squid and shrimp powder which were used to make the protein source for diet 1 (45% protein) while for diet 2, half the fish/squid/shrimp meal was replaced by soybean meal (45.2% protein) and diet 3

was casein-based microbound diet (powder diet with carrageenan as a binder)(44.5% protein). Shrimp fed diet 1 gave the best growth followed by diet 2 and significantly the lowest diet 3 fed shrimp. The reduced growth in diet 2 fed shrimp due to limiting amino acid particularly methionine (Hertrampf and Piedad-Pascual, 2000) in soybean meal whereas growth depression in diet 3 caused by the limiting threonine in casein (Mason and Castell, 2009).

The different growth responses due to the different quality fishmeal judged by raw material freshness were also found in shrimp reported by Ricque-Marie *et al*. (1998). Fishmeal produced from anchovy either fresh (12 h post capture), moderately fresh (25 h post capture) or stale (36 h post capture). Freshness was assessed through the total volatile nitrogen content in fish before process (TVN 14, 30 and 50 mg N/100 g fishmeal, respectively) and biogenic amines in fishmeal (histamine 28, 1850 and 4701 mg/kg, respectively and also with increasing content of cadaverine, putrescine and tyramine). Small *L. vannamei* (size 0.9 g) expressed significantly higher feed consumption and weight gain when fed diet containing fresh raw material fishmeal with 25% growth increasing compared with the moderately fresh and stale raw material treatments, whereas larger *L. vannamei* (size 1.5 and 7.6 g) did not show any significant response.

In-vitro protein digestibility using commercial diet induced enzyme fluctuated among treatments, whereas that from respective experimental diet induced enzyme was stable which might be the limitation on using *L. vannamei* midgut gland extract for measurement of *in-vitro* digestibility reported by Divakaran *et al*. (2004). As a result, *in-vitro* digestibility using experimental induced enzyme was adopted for digestibility assessment.

In-vitro and *in-vivo* protein digestibility reflected each other except grade 2 FM diet. Considering protein digestibility (*in-vitro* and *in-vivo*) excepting grade 2 FM showed the highest in premium grade FM (S1) diet followed by premium grade FM (S2), imported FM and grade 1 FM. Imported FM had high variation. Protein digestibility study in this experiment clearly demonstrated that fishmeal with same protein level had different protein digestibility. All treatments in the present study showed protein digestibility higher than 85% for *in-vivo* protein digestibility, while *in-vitro* digestibility was in range of 1.29-1.68 * 10^{-7} mole ala per 200 μL sample. The highest *in-vitro* protein digestibility was grade 2 FM diet, which had the lowest *in-*

vivo protein digestibility. The difference of digestibility values between the two techniques was probably due to the unique property of grade 2 FM. Grade 2 FM produced from surimi processing by-product which consisted of head, frame and offal which contained endogenous proteases that during storage could help digest that protein leading to partial digestion which might be highly digestible by *in-vitro* protein digestibility. The highest *in-vitro* protein digestibility of grade 2 FM diet was the lowest *in-vivo* protein digestibility. The dramatic difference of protein digestibility between two methods due to the measuring was on the different basis. The *in-vitro* protein digestibility was done in the reactor using hepatopancreas extracted crude enzyme from shrimp so that the results of this method showed amino acid product from the digestion process whereas the *in-vivo* method performed by trial using chromic oxide containing diet and measure quantity of protein in diets compared to that in feces.

The varying protein digestibility in response to different fishmeal quality in this study was similar to that reported by Cruz-Suarez *et al*. (2009) showing that apparent protein digestibility in *L. vannamei* of four soybean ingredients (full fat soy bean meal, solvent extracted soybean meal, soybean protein isolate and soybean protein concentrate) showed different capacity, that of soybean protein concentrate was significantly lower than in the other soybean products.

Trypsin gene expression in this study was related to amino acid balance of diet together with FM grade. Premium grade FM (S1) diet fed shrimp with a high ratio of 0.73 EAA/NEAA gave the highest gene expression. Shrimp fed premium grade imported FM (Chile), premium grade FM (S2) and grade 1 FM had similar gene expression and also had similar EAA/NEAA which was 0.68 and 0.69, respectively, except grade 1 FM. The equity of EAA/NEAA at 0.73 of premium grade FM and grade 1 FM with unequal of gene expression probably affected from quality including structure of that protein to form different FM quality and ultimately affected trypsin gene. Moreover, shrimp fed grade 2 FM with the lowest EAA/NEAA (0.65) was found un-expression of trypsin gene. Information in this area is shortage, but it might be due to the free amino acid provided from the digestion process induced the expression of trypsin gene as described in *Aedes aegypti* by Noriega and Wells (1999) where the free amino acid provided from the digestion stimulated translation of early trypsin gene which

was triggered for protein digestion and after that products from the digestion stimulate the late trypsin gene which the amino acid balance coupled with protein digestibility. Such mechanism might be the key factors affecting trypsin expression in *L. vannamei* in the present study. This needs further investigation. Trypsin gene expression alone cannot indicate the best growth, however. The best growth in this study clearly demonstrated that good amino acid balance together with protein digestibility induced trypsin gene expression with good feed intake responsible for good growth.

3.6 Conclusion

The results from this study clearly demonstrated that fishmeal quality affected growth performance in juvenile *L. vannamei* through mainly protein digestibility, feed intake and related with trypsin gene expression. Domestic premium grade fishmeal produced from a single species gave the best growth performance, the highest protein digestibility and also the highest trypsin gene expression.

Table 2 Amino acid composition of experimental diets (% of protein)

Ingredients	Experimental diets				
	Imported FM (Chile)	**Premium grade FM (S1)**	**Premium grade FM (S2)**	**Grade 1 FM**	**Grade 2 FM**
Arginine	4.20	4.43	2.72	5.60	4.55
Histidine	1.90	2.37	1.81	2.43	1.51
Isoleucine	1.94	2.29	1.45	3.14	1.98
Leucine	5.21	5.64	3.91	7.25	4.44
Lysine	4.73	5.11	3.54	5.45	4.07
Methionine	1.95	2.14	1.44	2.44	1.78
Phenylalanine	3.17	3.48	2.38	4.06	2.97
Threonine	3.02	3.31	2.28	3.70	2.91
Tryptophan	0.64	0.62	0.54	0.54	0.54
Valine	2.30	2.70	1.72	3.39	2.37
Alanine	4.72	4.83	3.57	5.56	4.52
Aspartic acid	7.06	7.36	5.28	7.58	6.19
Cystine	0.56	0.56	0.35	0.79	0.45
Glutamic acid	14.43	14.88	10.67	18.27	12.98
Glycine	5.27	5.15	3.69	6.51	6.51
Proline	4.63	4.86	3.45	5.58	5.33
Serine	3.57	3.78	2.64	4.02	3.31
Tyrosine	2.47	2.64	1.91	3.62	2.35
Essential amino acids	29.07	32.09	21.79	38.00	27.12
Non essential amino acids	42.70	44.07	31.57	51.94	41.64
EAA/NEAA	0.68	0.73	0.69	0.73	0.65

Table 3 Growth performance of *L. vannamei* fed diets with different fishmeal quality for 7 weeks

Experimental diets	**Final weight (g/shrimp)**	**Weight gain (g/shrimp)**	**SGR[2] (%/day)**	**Survival rate (%)**
A (Imported FM (Chile))	8.15±0.66[b1]	5.86±0.66[b]	2.53±0.16[b]	86.00±6.93
B (Premium grade FM (S1))	10.28±0.39[a]	7.99±0.38[a]	2.98±0.08[a]	88.00±9.24
C (Premium grade FM (S2))	9.35±0.58[a]	7.06±0.59[b]	2.81±0.13[a]	78.00±2.31
D (Grade 1 FM)	9.46±0.32[a]	7.16±0.31[a]	2.83±0.06[a]	81.00±3.83
E (Grade 2 FM)	9.16+0.18[a]	6.88+0.17[a]	2.82+0.06[a]	82.00±2.31

[1]Means in a column with the same superscript are not statistically different ($p>0.05$, n=4)

[2]Specific growth rate = $(\ln W_2 - \ln W_1/T_2 - T_1)*100$, W_1 = initial weight, W_2 = final weight, $T_2 - T_1$ = cultured period (days)

Table 4 Feed utilization efficiency of *L. vannamei* fed diets with different fishmeal quality for 7 weeks

Experimental diets	**Feed intake[2] (g/shrimp)**	**FCR[3]**	**PER[4]**	**PPV[5] (%)**
A (Imported FM (Chile))	8.79±0.52[c1]	1.51±0.09[a]	1.53±0.09[b]	27.41±1.59[b]
B (Premium grade FM (S1))	10.10±0.53[b]	1.28±0.09[b]	1.80±0.13[a]	32.83±2.38[a]
C (Premium grade FM (S2))	10.24±0.20[ab]	1.46±0.15[ab]	1.59±0.16[ab]	28.64±2.86[ab]
D (Grade 1 FM)	10.33±0.08[ab]	1.44±0.07[a]	1.60±0.07[ab]	28.75±1.33[ab]
E (Grade 2 FM)	11.06±0.46[a]	1.55±0.10[a]	1.49±0.09[b]	26.94±1.63[b]

[1]Means in a column with the same superscript are not statistically different ($p>0.05$, n=4)

[2]The reported feed intake was corrected for leaching loss

[3]Feed conversion ratio = feed intake (g)/weight gain (g)

[4]Protein efficiency ratio = weight gain (g)/protein intake (g)

[5]Productive protein value = (protein gain (g)/protein intake (g) * 100

Table 5 Proximate composition (%) of shrimp fed diets with different fishmeal quality for 7 weeks

Experimental diets	Moisture	Crude protein (Nx6.25)	Crude fat	Ash
A(Imported FM (Chile))	75.93±0.78[1]	74.30±1.23	8.65±0.26	10.27±0.22
B (Premium grade FM (S1))	75.54±0.51	74.55±0.67	8.14±0.13	10.66±0.36
C (Premium grade FM (S2))	75.93±1.11	74.82±0.62	8.40±0.18	10.25±0.18
D (Grade 1 FM)	75.90±1.03	74.69±1.09	8.05±0.11	10.28±0.45
E (Grade 2 FM)	75.70±1.02	74.45±0.30	8.91±0.21	9.85±0.32

[1]Means are not statistically different in all parameters (p>0.05, n=4)

Table 6 *In-vitro* and *in-vivo* protein digestibility of shrimp fed diets with different fishmeal quality

Experimental diets	AG liberated by commercial feed induced enzyme (10^{-7} mole ala/ 200 μL sample)	AG liberated by experimental feed induced enzyme (10^{-7} mole ala/ 200 μL sample)	*In-vivo* digestibility (%)	Specific trypsin activity (unit/min/mg. protein)	Feces (g/2 weeks)
A (Imported FM (Chile))	0.17±0.01 [bc1]	1.29±0.43	90.83±0.30 [a]	1.29±0.04	2.53±0.71 [b]
B (Premium grade FM (S1))	0.31±0.15 [c]	1.64±0.06	92.06±0.42 [a]	1.37±0.02	3.62±1.07 [b]
C (Premium grade FM (S2))	1.55±0.15 [ab]	1.61±0.06	90.56±0.54 [a]	1.32±0.13	3.13±0.56 [b]
D (Grade 1 FM)	1.07±0.77 [ab]	1.47±0.02	88.75±0.66 [b]	1.29±0.04	4.33±1.01 [b]
E (Grade 2 FM)	2.07±0.16 [a]	1.68±0.10	85.07±0.13 [c]	1.29±0.01	7.88±1.85 [a]

[1]Means in a column with the same superscript and without superscript are not statistically different (p>0.05, n=3)

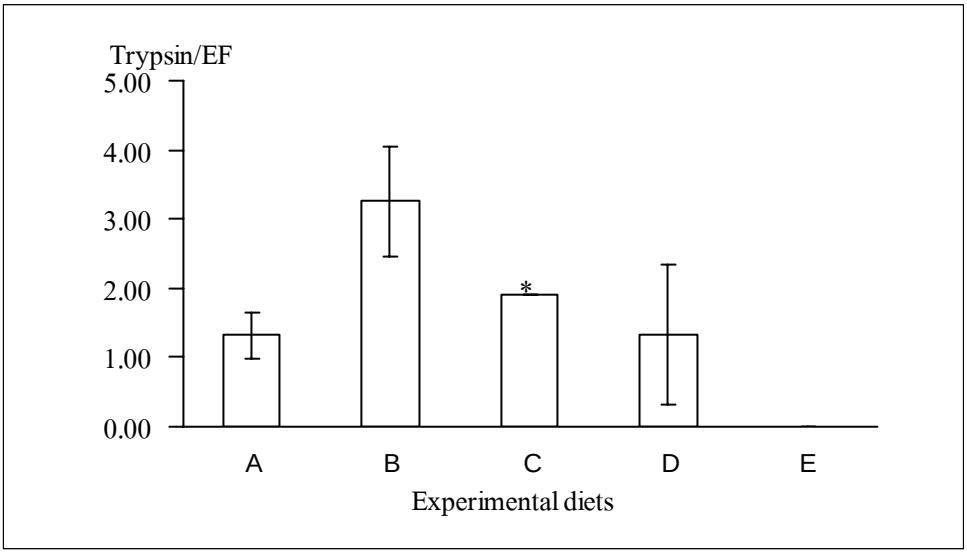

Fig. 3 Trypsin gene expression of *L. vannamei* midgut gland fed diets. Different letters indicate statistical differences between groups (n=2 except *, n=1) (A, imported FM (Chile); B, Premium grade FM (S1); C, Premium grade FM (S2); D, Grade 1 FM; E, Grade 2 FM)

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CHAPTER 4

Hemoglobin Powder Substituted for Fishmeal on Growth Performance, Protein Digestibility and Trypsin Gene Expression in Pacific White Shrimp (*Litopenaeus vannamei*)

4.1 Abstract

The rising price and demand of fishmeal which is the most important protein source in shrimp feed has caused a search for an alternative protein source. Hemoglobin powder (HE) is produced by separating hemoglobin from plasma of farm animal un-coagulant blood. HE contains high protein content but low lipid content thus it has a potential for fishmeal substitution in animal feeds.

A six week feeding trial was carried out to investigate effects of HE substituted for fishmeal protein on growth performance, protein digestibility and trypsin gene expression. Six diets with 0%, 12.5%, 25%, 50%, 75% and 100% of HE replacing fishmeal protein were fed four times daily to six groups of shrimp with an average initial weight of 3.53 g/shrimp. Growth of shrimp dramatically decreased with increasing level of HE substitution. Although the 12.5% HE substitution caused significantly lower final weight, weight gain, SGR, feed intake, PER and PPV in comparison with the control diet, FCR of this diet was not statistically different ($p<0.05$).

The activity of trypsin of shrimp was similar among the groups fed diets with HE substitution not higher than 50% ($p>0.05$). *In-vitro* and *in-vivo* protein digestibility coefficients of 12.5% HE substitution were significantly lower than those of the control group which the trypsin gene expression of shrimp fed 12.5% HE substituted diet was the highest.

4.2 Introduction

Pacific white shrimp, *Litopenaeus vannamei*, is an exotic species that is becoming an economical important species in Thailand because of its ability to adapt in a wide range of salinities and temperatures and to cope with diseases under high intensity culture conditions. Growing shrimp production worldwide increases feed demand, which directly raises

a higher demand for shrimp feed ingredients, particularly fishmeal. Fishmeal is the main protein source in many aquatic feeds due to its suitable amino acid profile and palatability. While fishmeal production is stable or even decreasing, the rising price and increasing demand for fishmeal in animal production systems are expected to cause a shortage of the meal in the future unless replacements are found. Thus the shrimp feed industry is searching for other suitable protein sources.

The protein sources which are used to substitute for fishmeal in shrimp feeds are plant protein, such as soybean meal, and terrestrial processing by-product protein, such as meat and bone meal, poultry by-product, blood meal and hemoglobin meal (Abery *et al*., 2002; Bureau *et al*., 1999; Forster *et al*., 2003; Lim and Dominy, 1990; Tacon and Akiyama, 1997) but the substituted levels varied depending on protein sources. Inclusion of different protein sources at the same protein level may not give the same muscle growth because of disparity in protein quality and digestibility. From an industrial point of view, the highest substitution level for fishmeal is preferred to lower the production cost. However, fishmeal replacement diet may not support good growth due to the inferior amino acid profile and protein digestibility of the alternative protein sources used in the diets. Hemoglobin powder is a candidate as an alternative protein source due to its high protein content and digestibility with high lysine and leucine content (Hertrampf and Piedad-Pascual, 2000).

This study was therefore carried out to investigate fishmeal substitution with hemoglobin powder on growth performance, *in-vitro* and *in-vivo* protein digestibility and trypsin gene expression.

4.3 Materials and Methods

4.3.1 Experimental diets and leaching tests

Fishmeal used in this study was a premium grade (65.36% protein) purchased from a fishmeal plant (Pattani Fishmeal Industry Co., Ltd.) in Pattani province, Thailand. Hemoglobin powder (84.16% protein) was donated by a feed company (Inteqe Feed Co., Ltd.).

Six diets were formulated to contain protein and lipid at 45% crude protein and

Table 7 Composition (g/100 g), proximate composition (% as fed basis) and leaching loss (dry matter basis) of experimental diets

Ingredients	Experimental diets					
	control	**12.5% HE**	**25% HE**	**50% HE**	**75% HE**	**100% HE**
Fishmeal (65.36% protein)	44	38.5	33	22	11	-
Hemoglobin powder (HE) (84.16% protein)	-	4.27	8.54	17.09	25.63	34.17
Squid meal	8	8	8	8	8	8
Wheat flour	20	20	20	20	20	20
Rice flour	15	15	15	15	15	15
Wheat gluten	6	6	6	6	6	6
Lecithin	2	2	2	2	2	2
Tuna fish oil	-	0.5	1	2	3	4
Vitamin mix[1]	2	2	2	2	2	2
Vitamin C	0.1	0.1	0.1	0.1	0.1	0.1
Trace mineral mix[2]	0.5	0.5	0.5	0.5	0.5	0.5
Calcium phosphate	0.2	0.2	0.2	0.2	0.2	0.2
BHT	0.02	0.02	0.02	0.02	0.02	0.02
Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5
CMC	1	1	1	1	1	1
Cellulose	0.68	1.41	2.14	3.59	5.05	6.51
Proximate composition (% as fed basis) and leaching loss (dry matter basis)						
Protein	45.89	46.75	46.51	44.83	45.16	45.79
Lipid	7.89	7.53	7.48	7.67	8.38	7.56
Ash	7.64	6.76	6.19	4.96	3.61	2.27
Leaching loss (%)	12.07	12.45	11.23	8.43	8.70	6.86

[1] Vitamin mix (g/kg vitamin mix): thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0%, biotin 0.05, folic acid 0.18, cyanocobalamine 0.002, choline chloride 100, inositol 5.0, menadione 2.0, retinol acetate (20,000 IU/g) 5.0, cholecalciferol (400,000 IU/g) 0.002, DL-alpha-tocopheryl acetate (250 IU/g), wheat flour 865.266

[2] Mineral mix (g/100 g mineral mix): cobalt chloride 0.004, cupric sulfate pentahydrate 0.250, ferrous sulfate 4.0, magnesium heptahydrate 28.398, manganous sulfate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, wheat flour 53.428

8% lipid with hemoglobin powder substituted for fishmeal at 0, 12.5, 25, 50, 75 and 100% (Table 7). Diet preparation process is the same as described in previous experiment in Chapter 3. The proximate composition of ingredients and experimental diets was determined (AOAC, 1995). The amino acid profile of diets was determined by HPLC (AOAC, 1995).

Diet leaching test was performed using three replicates according to the method modified from Aquacop (1978) and Cruz-Suarez *et al*. (2001) as describe in the Chapter 3.

4.3.2 Growth trial

Shrimp, culture and feeding

Juvenile *L. vannamei* shrimp were nursed at Aquatic Science Research Station, Satun province, Thailand until used. The shrimp were stocked into 24 glass aquaria (45*45*115 cm) containing 200 L of natural seawater (water flow rate 33.26 L/h, water temperature 26-30 $^{\circ}$C, salinity 29-33 ppt) and acclimatized to the experimental conditions for one week. Twenty shrimp with individual initial weight of 3.53$\pm$0.06 g were then selected and randomly distributed into each of 24 glass aquaria and fed with experimental diets. Feeding was done by hand to satiation determined by slow or no response to the diet, 4 times daily at 8.00 am, 12.00 am, 5.00 pm and 10.00 pm for 6 weeks. Uneaten feed was collected for feed intake correction.

Sampling

At the end of feeding period, ten shrimp from each aquarium were sampled. Six shrimp were used for proximate composition analysis. Two shrimp were decapitated and the hepatopancreas were fixed in TRIzol reagent and kept at -80 $^{\circ}$C until used for trypsin gene expression analysis, while the final two shrimp were decapitated and hepatopancreas were taken for enzyme extraction for *in-vitro* protein digestibility. The remaining shrimp were left in aquaria for 30 days for *in-vivo* protein digestibility determination.

4.3.3 *In-vitro* protein digestibility

In-vitro protein digestibility of experimental diets was determined using crude enzyme extract from the hepatopancreas from the shrimp in the experiments described above.

The crude enzyme extract were prepared and the *in-vitro* protein digestibility study was performed using the method modified from Bassompierre (1997) as described in Chapter 3.

4.3.4 *In-vivo* protein digestibility

Apparent digestibility coefficient (ADC) of crude protein in diets was measured using the same method as described in Chapter 3.

4.3.5 Trypsin gene expression

Trypsin gene expression was studied using 2 steps RT-PCR as described in Chapter 3.

4.3.6 Statistical Analysis

Growth performance, survival rate, feed utilization efficiency, protein digestibility and trypsin gene expression data were analyzed using analysis of variance to determine if significant differences exist among treatment means. The Tukey's HSD test was used to determine significant differences between treatments. Final weight was analyzed using linear regression procedures with fishmeal replacement level as the independent variable. A 5% error rate for significance was used for all analyses.

4.4 Results

4.4.1 Amino acid composition of experimental diets

The amino acid profile of 6 diets (Table 8) produced from different levels of HE replacement of fishmeal gave varying amino acid composition, reflecting the amino acid profiles of the two protein sources. The high glutamic amino acid (22.95% of protein) content in the control diet decreased with the increasing HE levels, with the 100% HE diet having the lowest content (16.32% of protein). Moreover, arginine, glycine, methionine, proline, isoleucine, tyrosine and lysine attended to decline. In contrast, leucine, phenylalanine, aspartic acid, alanine,

serine, histidine, valine, threonine and tryptophan increased with HE increment. In addition, EAA/NEAA balance was changed and the proportion of leucine and isoleucine dramatically changed with the increasing HE.

4.4.2 Survival rate

The survival rate of shrimp fed the control diet, 12.5, 25, 50, 75 and 100% HE were 96.25, 95.00, 83.75, 95.00, 91.25 and 96.25%, respectively (Table 9).

4.4.3 Growth and feed utilization

Shrimp fed the control diet gave the significantly highest final weight, weight gain, SGR, PER and PPV (Tables 9 and 10) which were 10.38, 6.88, 2.59, 1.61 and 30.40, respectively. Feed intake of the control treatment was also significantly ($p<0.05$) higher while FCR was the lowest with values of 9.33 and 1.44, respectively. Final weight, weight gain, SGR, PER and PPV of shrimp fed diets decreased with increasing levels of HE substitution. HE levels significantly affected final weight with $R^2 = 0.8446$ (Fig. 4).

Feed intake of shrimp fed diets with hemoglobin replacement was lower than that of the control shrimp and was the lowest in 100% replacement. Replacement with HE caused higher FCR although the trend of increment was not correlated with levels of replacement but 100% replacement diet gave the highest FCR.

Proximate analysis of final shrimp (Table 11) showed the highest ash content in those fed the 100% HE diet while protein and lipid content were not significantly different ($p>0.05$) among treatments.

4.4.4 Protein digestibility

In-vitro and *in-vivo* protein digestibility coefficients of 12.5% HE substituted diet were the lowest without significant difference ($p>0.05$) than that of other groups (Table 12). *In-vitro* protein digestibility of diets noticeably related with the levels of HE substitution

coincided with *in-vivo* protein digestibility, however regression between *in-vitro* and *in-vivo* protein digestibility was very low (R^2 = 0.0004).

4.4.5 Gene expression

Trypsin gene expression of shrimp fed 12.5% HE diet (Figure 5) was the highest, whereas the protein digestibility of that treatment was the lowest. However, the expression of this gene was not related with the levels of hemoglobin substitution. Regardless of 12.5% HE treatment, the other HE diets showed the trend of inversed effect of inclusion levels on trypsin gene expression.

4.5 Discussion

Growth responses and feed utilization decreased with increasing levels of HE were due to two main causes. First was reduction in feed intake indicating unpalatibility of HE. Feed intake of shrimp fed 100% HE diet was only 5.91 g/shrimp whereas that of the control group was 8.20 g/shrimp.

The increment of hemoglobin replacement caused the reduction of a non essential amino acid, glutamic acid, which is a palatability agent in food and feed, particularly for shrimp and fish (D'Abramo, 1997). Yousif *et al*. (1996) reported reduced feed intake of tilapia due to blood meal substituted for fishmeal. FCR reflected feed utilization ability of ingested protein by test animals. The present study also demonstrated that single hemoglobin protein as a replacer in high quantity strongly showed unaccepted by shrimp although that level of inclusion enhanced the protein digestibility and lowering leaching loss.

The second cause of a lower and feed utilization of shrimp was imbalanced amino acid profile of the HE diets. Increasing HE levels resulted in reduction of dietary arginine, isoleucine and methionine while increasing leucine levels leading to an imbalance of branched-chain amino acid (BCAA), leucine, isoleucine and valine. The higher HE substitution level, the higher the imbalance of BCAA, particularly those between leucine and isoleucine. Arginine and

methionine contents in every HE substituted diet were lower than the recommended level for *L. vannamei* which are 5.8 and 2.4% of protein, respectively (Akiyama *et al*., 1991) whereas those of the 100% HE diet were only 3.38 and 1.25% of protein, respectively.

The present study is the first report on hemoglobin substitution for fishmeal in shrimp diets. Capability of hemoglobin substituted for fishmeal in *L. vannamei* in this study was lower than those in juvenile Japanese eel as reported by Lee and Bai (1997a) where the level of hemoglobin inclusion in diet can be up to 50% without essential amino acid supplementation and up to 75% with essential amino acid supplementation without an adverse effect on growth performance and feed utilization. A similar result was reported in Nile tilapia where Lee and Bai (1997b) successfully replaced FM with HE up to 50%. Martinez-Llorens *et al*. (2008), in contrast, reported that juvenile and on-growing gilthead sea beam fed diets containing hemoglobin powder substituted for fishmeal up to 15% showed a reduction in growth, which is similar to the level of replacement in this study. The difference among these species could be due to differences in essential amino acid requirements of animals. Substitution for fishmeal with hemoglobin powder in high quantity may result in elevating Fe in the body resulting in pathological effects. Despite no pathological evidence in this study, shrimp fed 100% HE diet had the highest ash content which was similar to those observed in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum) when fed diets with spray-dried blood meal replacing 17.5% fishmeal (Fowler and Banks, 1976). Blood meal is known to contain high levels of iron (2,769 mg/kg) and zinc (306 mg/kg) (NRC, 1993). In general, the responses of different species to blood meal incorporated diets have been varied.

Whole blood meal is closely related to hemoglobin powder although it contains blood plasma. Many researchers have studied replacement of blood meal for fishmeal either in shrimp and fish. Dominy and Ako (1988) reported that the 15% blood meal products (ring-dry blood meal, sun-dried blood meal, sun-dried blood meal added crystalline methionine or sun-dried blood meal accompanied covalently linked methionine) could be substituted for marine protein in diets fed to *L. vannamei* without any effect on survivor, growth performance and feed utilization as compared to the control groups. However, Abery *et al*. (2002) found that Murray cod *Maccullochella peelii peelii* (Mitchell) receiving a diet containing 8% blood meal gave

significantly lower final weight and significantly higher FCR compared to those fed the control diet. In addition, Martinez-Llorens *et al*. (2008) reported that blood meal can substitute for fishmeal up to 15% with no effect on growth performance, while hemoglobin substituted at the same level caused a reduction in fish growth (Martinez-Llorens *et al*., 2008).

Both *in-vitro* and *in-vivo* digestibility were high with increasing HE levels. The results of this study demonstrate that HE used in the diets were easily digested by white shrimp, which was similar to those reported in Australian snapper *Pagrus auratus* (Booth *et al*., 2005). The ingested and digested diets nevertheless could not be utilized by shrimp because of the elevating degree of amino acid imbalance and deficiencies associated with the HE levels included in diets.

Growth responses were not reflected by the trypsin gene expression in the present study. In 12.5% HE fed shrimp with the growth response second to the control group gave the highest gene expression while *in-vitro* and *in-vivo* digestibility were the lowest. The results might be the responses to the better amino acid profile of the diet rather than to the HE inclusion level. Another explanation is the physiological response of compensation for the more difficult digested diet as compared to higher digestible HE incorporated diet. Meanwhile, shrimp fed the 25% and 50% HE diets showed gene expression equal to that of the control diet (0%HE) whereas shrimp fed the 75% and 100% HE diets were second to the 12.5% diet. This could be due to the replacement of the HE at 25% and 50% had equal digestibility resulting in the similar gene expression while 75% and 100% HE diets had affected the palatability of that diet resulted in unsatisfying feed intake and finally affected physiological functions that is the high gene expression took place in this case due to the hunger. It may be deduced that the quality and unpalatibility of diets, diet digestibility and the physiological responses of shrimp resulted in the low growth response of shrimp fed high levels of HE. The relationship between diet quality and gene expression have not been reported elsewhere. Muhlia-Almazan *et al*. (2003) studied trypsin gene expression in response to dietary protein levels in *L. vannamei* and found that 30% protein fed shrimp showed the highest gene expression as compared to 15% and 50% protein fed shrimp. In addition, Sanchez-Paz *et al*. (2003) reported that trypsin-encoding RNA levels were strongly influenced by starvation by *L. vannamei*, resulting in an increase after 24 h of starvation.

The gelling property of HE can be observed during feed production of the present study. The 100% HE diet had good water stability with leaching loss of only 6.86% while that of the control diet was 12.07%. Incorporation of HE in shrimp diets improves the pellet binding capacity (Hertrampf and Piedad-Pascual, 2000) besides the alternative protein source.

From an economic point of view, HE could be considered as an alternative protein source for fishmeal due to its high protein content, good binding property and availability. However, the appropriate level of substitution in shrimp diets is to be investigated and use in combination with other protein sources is recommended to mitigate indispensible amino acid imbalances.

4.6 Conclusion

This study clearly demonstrates the inability of *L. vannamei* to utilize HE even at 12.5% replacement for fishmeal protein. Further study on the appropriate level of HE in diet for *L. vannamei* needs to be conducted.

Table 8 Amino acid composition of experimental diets (% of protein)

Amino acid	Experimental diets					
	Control	**12.5% HE**	**25%HE**	**50%HE**	**75%HE**	**100%HE**
Arginine	5.46	5.33	5.20	4.42	3.90	3.38
Histidine	4.19	4.12	4.30	4.92	5.28	5.64
Isoleucine	1.35	1.53	1..40	0.94	0.74	0.53
Leucine	5.69	6.71	7.28	7.31	8.12	8.93
Lysine	6.05	6.11	6.19	5.88	5.79	5.71
Methionine	2.43	2.30	2.09	1.84	1.54	1.25
Phenylalanine	4.00	4.60	5.04	5.22	5.83	6.44
Threonine	3.30	3.82	3.84	3.53	3.64	3.76
Tryptophan	0.70	0.78	0.71	0.87	0.96	1.05
Valine	2.01	2.58	2.75	2.50	2.74	2.99
Alanine	7.58	7.49	7.60	8.49	8.94	9.39
Aspartic acid	9.45	10.32	10.46	10.54	11.08	11.62
Cystine	1.09	1.14	1.13	1.00	0.96	0.92
Glutamic acid	22.95	20.15	18.66	19.63	17.98	16.32
Glycine	7.67	7.28	7.42	7.00	6.66	6.33
Proline	7.01	6.51	6.70	6.45	6.16	5.88
Serine	5.04	5.55	5.43	5.82	6.20	6.59
Tyrosine	4.02	3.69	3.80	3.65	6.46	3.28
Essential amino acids	35.18	37.88	38.80	37.43	38.55	39.67
Non essential amino acids	64.82	62.12	61.20	62.57	61.45	60.33
EAA/NEAA	0.54	0.61	0.63	0.60	0.63	0.66

Table 9 Growth of *L. vannamei* fed diets with hemoglobin powder substituted for fishmeal over 6 week feeding trial

Experimental diets	Final weight (g/shrimp)	Weight gain (g/shrimp)	SGR[2] (%/day)	Survival rate (%)
Control	10.38±0.45[a1]	6.88±0.41[a]	2.59±0.08[a]	96.25±4.79
12.5% HE	8.76±0.29[b]	5.13±0.34[b]	2.13+0.12[b]	95.00±4.08
25% HE	8.51±0.40[b]	4.95±0.42[b]	2.07±0.13[bc]	83.75±10.31
50% HE	7.67±0.28[c]	4.15±0.24[bc]	1.85±0.06[cd]	95.00±4.08
75% HE	7.19±0.23[c]	3.65±0.19[c]	1.68±0.06[d]	91.25±7.50
100% HE	5.79±0.23[d]	2.26±0.26[d]	1.17±0.12[e]	96.25±2.50

[1] Means in a column with the same superscript are not statistically different ($p>0.05$, $n=4$)

[2] Specific growth rate = $(\ln W_2 - \ln W_1)/(T_2-T_1)*100$, W_1 = initial weight, W_2 = final weight, T_2-T_1 = cultured period (days)

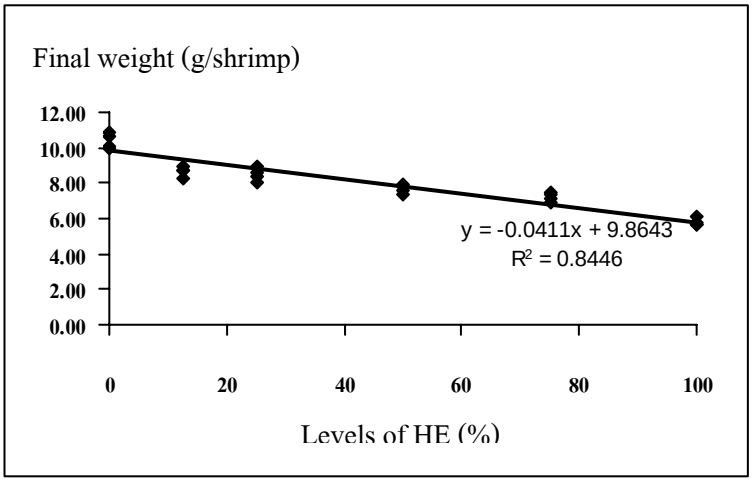

Figure 4 Regression between final weight of shrimp and levels of HE substituted for fishmeal

Table 10 Feed utilization efficiency of *L. vannamei* fed diets with hemoglobin powder (HE) substituted for fishmeal over 6 week feeding trial.

Experimental diets	Feed intake (g/shrimp)	FCR[2]	PER[3]	PPV[4] (%)
Control	8.20±0.37[a1]	1.19±0.09[d]	1.83±0.14[a]	34.57±2.79[a]
12.5% HE	7.32±0.18[b]	1.43±0.10[cd]	1.50±0.10[b]	27.27±0.03[b]
25% HE	7.55±0.30[b]	1.53±0.09[c]	1.44±0.08[b]	26.24±1.62[b]
50% HE	7.01±0.29[b]	1.70±0.16[c]	1.32±0.13[bc]	24.95±3.33[b]
75% HE	7.24±0.11[b]	1.99±0.11[b]	1.12±0.06[c]	20.67±1.70[bc]
100% HE	5.91±0.35[c]	2.63±0.18[a]	0.84±0.06[d]	14.34±1.71[c]

[1] Means in a column with the same superscript are not statistically different ($p>0.05$, $n=4$)

[2] Feed conversion ratio = feed intake (g)/weight gain (g)

[3] Protein efficiency ratio = weight gain (g)/protein intake (g)

[4] Productive protein value = (protein gain (g)/protein intake (g)) * 100

Table 11 Proximate composition (%) of *L. vannamei* fed diets containing various levels of hemoglobin powder (HE) for 6 weeks (dry matter basis)

Composition	Experimental diets					
	control	12.5% HE	25% HE	50% HE	75% HE	100% HE
Moisture	75.00±0.60[b]	75.63±0.61[ab]	75.54±0.34[ab]	75.55±0.45[ab]	75.87±0.31[ab]	76.55±0.31[a]
Crude protein	72.22±0.93	73.62±1.11	73.40±1.65	73.04±1.39	71.96±0.12	71.57±1.16
Crude fat	5.73±0.71	4.20±0.17	6.83±1.12	3.56±1.23	3.83±0.85	4.27±0.95
Ash	11.94±0.37[ab]	11.74±0.15[b]	11.63±0.34[b]	11.65±0.33[b]	12.13±0.80[ab]	13.05±0.48[a]

[1] Means in a row with the same superscript are not statistically different ($p>0.05$, $n=4$)

Table 12 *In-vitro* and *in-vivo* protein digestibility of shrimp fed diets substituted fishmeal with hemoglobin powder (HE) at varying levels

Experimental diets	**AG liberated by experimental feed induced enzyme (10^{-7} mole ala/ 200 μL sample)**	***In-vivo* digestibility (%)**	**Feces (g wet weight/ shrimp/29 days)**
Control	1.61±0.19[1]	86.03+1.09	4.33±0.56
12.5 % HE	1.18±0.20	83.25+0.34	4.62±0.43
25 % HE	1.40±0.31	85.70+0.44	4.59±1.46
50 % HE	1.59±0.16	84.77+1.61	4.96±1.19
75 % HE	1.52±0.42	85.36+1.61	3.70±0.53
100 % HE	1.82±0.35	83.54+0.30	3.70±0.58

[1] Means are not statistically different in all parameters ($p>0.05$, $n=3$)

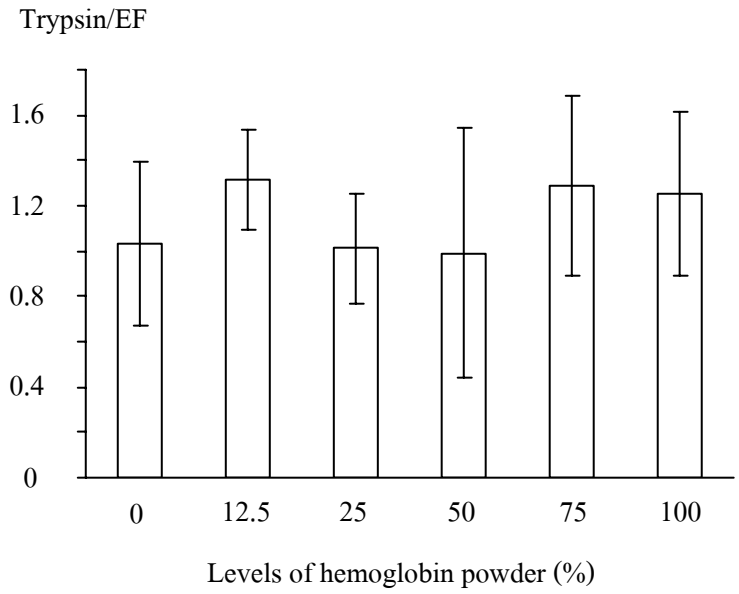

Figure 5 Trypsin gene expression of shrimp fed varying diets containing fishmeal replacement with different levels of hemoglobin powder, average gene expression from 3 replicates.

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CHAPTER 5

Fishmeal Substituted with Soybean Meal and Hemoglobin Powder on Growth Performance, Protein Digestibility and Trypsin Gene Expression in Pacific White Shrimp (*Litopenaeus vannamei*)

5.1 Abstract

An eight week feeding trial was carried out to investigate effects of hemoglobin powder (HE) substituted for fishmeal (FM) using basal diet containing FM and soybean meal (SBM) at 60:40. Six experimental diets with HE replacing FM at 0, 6.73%, 13.52%, 19.80%, 19.80% + amino acid and 26.53% + amino acid supplementation were formulated. A reference diet with FM as a sole source of protein is also included in the study. Each diet was fed four times daily to triplicate groups of shrimp with an average initial weight of 1.39 g/shrimp for 8 weeks. Growth performance, protein digestibility and trypsin gene expression were measured. Growth performance of shrimp fed the control diet and three combination protein diets at every level of HE substituted for FM including amino acid supplemented diets was lower than that of shrimp fed the reference diet. Among HE substituted diets, final weight, weight gain and SGR showed a trend of reduction with respected to an increasing level of HE substitution regardless of amino acid supplementation. Feed intake of all treatments was similar including that of the reference diet. FCR increased with the increasing levels of HE substitution regardless of amino acid supplementation, whereas FCR of the reference diet was significantly the lowest. PER and PPV decreased with increasing levels of HE substitution. Supplementation of amino acid resulted in slightly better PER and PPV in 19.80% HE+ amino acid diet but was still lower than the control diet however, 26.53% HE+ amino acid diet was not effective in improving protein utilization. The reference diet showed significantly the highest PER and PPV.

In-vitro and *in-vivo* protein digestibility coefficients showed a similar trend and reflected each other. Protein digestibility was the highest in the reference diet but decreased with the substitution of FM with other protein sources. HE substituted for FM resulted in reducing apparent crude protein digestibility which was the lowest in the diet with 26.53% HE regardless of amino acid supplementation.

Trypsin gene expression fluctuated among treatments whereas trypsin gene expression of 6.73% HE diet was consistently the lowest.

5.2 Introduction

Fishmeal (FM) is a preferred ingredient in balanced rations for shrimp because of its high protein content, essential amino acid composition, particularly lysine and methionine, n-3 fatty acids, mineral content and palatability and digestibility (Alvarez *et al*., 2007). Estimated demand for FM will increase from 372,000 to 485,000 tons during the present decade, solely for manufacturing feedstuffs for shrimp (Barlow, 2000). FM is also used as a protein source for terrestrial animal production. Substitutes for FM, which provide adequate nutrition and are economically feasible, need to be found (Alvarez *et al*., 2007). To diminish dependence on FM, many strategies are used such as using single alternative protein sources with amino acid supplementation and combination of protein sources. Replacement with combined protein sources is a reasonable strategy to balance amino acid profile of the combined protein.

Soybean meal (SBM) is a digestible protein source and has good amino acid profile, although methionine and cystine are limited while arginine and phenylalanine are in good supply (Hertrampf and Piedad-Pascual, 2000).

Hemoglobin powder (HE) is a blood product containing high protein but low fat. The high protein digestibility, high mineral content (particularly calcium) and good binding property are key factors to consider HE application in shrimp feed. As with blood meal, HE is low in isoleucine, cystine and methionine but rich in leucine, lysine and valine content (Hertrampf and Piedad-Pascual, 2000). The high digestible protein with the unique amino acid profile between SBM and HE might help balance amino acid profile and promote good growth in cultured animals. Growth responses of this protein combination have not been published elsewhere while the combination of SBM with other protein sources has been published.

Combined plant protein sources at all levels of SBM (32.5, 34.9, 37.2 and 39.6%) and corn gluten meal (0, 1.7, 3.2 and 4.8%) was successful for fishmeal substitution at 9% in juvenile *L. vannamei* reared in pond (Amaya *et al*., 2007). Combination of SBM (35, 46.8

and 79.8%) and brewer's grains with yeast (0, 5 and 30%) can totally replace FM (0, 10 and 25% fishmeal) for juvenile Australian red claw crayfish *Cherax quadricarinatus*. Using HE in combined protein sources was unpublished while single protein replacement was employed in many species at varying levels. Juvenile Japanese eel and Nile tilapia can utilize HE at 50% replacement for FM while impaired growth was noted, even at 15% replacement in juvenile gilthead sea beam (Lee and Bai, 1997a; Lee and Bai, 1997b; Martinez-Llorens *et al*., 2008).

This study was carried out to study the effect of HE substituted for FM in combination of three protein sources (FM, SBM and HE) on growth performance, *in-vitro* and *in-vivo* protein digestibility and trypsin gene expression.

5.3 Materials and Methods

5.3.1 Experimental diets and leaching tests

Fishmeal (FM) used in this study was a premium grade (67.52% protein) purchased from a FM plant (Pattani Fishmeal Industry Co., Ltd.) in Pattani province, Thailand. Hemoglobin powder (HE) (86.05%) was donated by a feed company (Inteqe Feed Co., Ltd.).

Seven diets were formulated to contain protein and lipid at 45% crude protein and 8% lipid and prepared as follow: reference diet using fishmeal as a single protein source with no soybean meal; control diet prepared using combination of fishmeal and soybean meal at 60:40; diets which were substituted for fishmeal in combination protein with HE at 6.73%, 13.52%, 19.80%; amino acid supplement diets which were substituted fishmeal in combination protein with hemoglobin powder at 19.80% and 26.53% accompanied with amino acid supplement. Diet preparation process is the same as described in Chapter 3. The proximate composition of ingredients and experimental diets was determined (AOAC, 1995). The amino acid profile of diets was determined by HPLC (AOAC, 1995).

Diet leaching test was performed using three replicates according to the method modified from Aquacop (1978) and Cruz-Suarez *et al*. (2001) as described in Chapter 3.

Table 13 Composition (g/100 g), proximate composition (as fed basis) and leaching loss (dry matter basis) of experimental diets

Ingredients	Experimental diets						
	Reference	Control	6.73% HE	13.52% HE	19.80% HE	19.80% HE+aa	26.53% HE+aa
Fishmeal (67.52% protein)	44	26.40	24.60	22.90	21.10	21.10	19.40
Hemoglobin meal (86.05% protein)	-	-	1.40	2.80	4.10	4.10	5.50
Soybean meal (41.46% protein)	-	28.70	28.70	28.70	28.70	28.70	28.70
Squid meal	8	8	8	8	8	8	8
Wheat flour	18	18	18	18	18	18	18
Rice flour	2.40	2.40	2.60	1.80	3.10	1.30	0
Wheat gluten	6	6	6	6	6	6	6
Lecithin	2	2	2	2	2	2	2
Tuna fish oil	1.30	2.40	2.50	2.70	2.80	2.80	3.30
Isoleucine	-	-	-	-	-	0.11	0.148
Methionine	-	-	-	-	-	0.026	0.035
Vitamin mix[1]	2	2	2	2	2	2	2
Vitamin C	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mineral mix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Calcium phosphate	0.20	0.20	0.20	0.20	0.20	0.20	0.20
BHT	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Cholesterol	0.50	0.50	0.50	0.50	0.50	0.50	0.50
CMC	1	1	1	1	1	1	1
Cellulose	13.47	1.27	1.37	2.27	1.37	3.03	4.09
98% Chromic oxide	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Proximate composition (as fed basis) and leaching loss (dry matter basis) of experimental diets							
Protein	46.32	45.65	45.94	45.63	47.04	44.23	45.01
Lipid	6.36	6.47	5.50	5.18	7.43	7.93	6.19
Ash	8.07	7.80	7.55	7.50	7.22	7.23	6.93
Leaching loss (%)	14.70	19.26	19.90	18.78	16.43	17.97	18.01

[1] Vitamin mix (g/kg vitamin mix): thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0%, biotin 0.05, folic acid 0.18, cyanocobalamine 0.002, chloline chloride 100, inositol 5.0, menadione 2.0, retinol acetate (20,000 IU/g) 5.0, cholecalciferol (400,000 IU/g) 0.002, DL-alpha-tocopheryl acetate (250 IU/g), wheat flour 865.266

[2] Mineral mix (g/100 g mineral mix): cobalt chloride 0.004, cupric sulfate pentahydrate 0.250, ferrous sulfate 4.0, magnesium heptahydrate 28.398, manganous sulfate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, wheat flour 53.428

5.3.2 Growth trial

Shrimp culture and feeding

Juvenile *L. vannamei* shrimp were nursed at Aquatic Science Research Station, Satun province until used. The shrimp were held in 28 glass aquaria (45*45*115 cm) containing 200 L of natural seawater (temperature 26-30 °C, salinity 9-15 ppt) with 70% water exchange at 7.00 am daily and acclimatized to the experimental conditions for one week. Twenty shrimp with individual initial weight of 1.39±0.03 g were then selected and randomly distributed into each of 28 glass aquaria. Feeding was done by hand to satiation determined by slow or no response to the diet, 4 times a day at 8.00 am, 12.00 am, 5.00 pm and 10.00 pm for 8 weeks. Uneaten feed was collected for feed intake correction.

Sampling

At the end of the feeding period, 10 shrimp from each aquarium were sampled. Six shrimp were used for proximate composition analysis. Two shrimp were decapitated and the hepatopancreas were fixed in TRIzol reagent and kept at -80 °C until use for trypsin gene expression analysis. Another final two shrimp were decapitated and hepatopancreas were taken to extract enzyme, then pooled for replicated aquaria and used for *in-vitro* protein digestibility study. Feces from each treatment were collected throughout the culture period for *in-vivo* protein digestibility determination.

5.3.3 *In-vitro* protein digestibility

In-vitro protein digestibility of experimental diets was determined using crude enzyme extract from the hepatopancreas from the shrimp in the experiments described above. The crude enzyme extracts were prepared and the *in-vitro* protein digestibility study was performed using the method modified from Bassompierre (1997) as described in Chapter 3.

5.3.4 *In-vivo* protein digestibility

Apparent digestibility coefficients (ADC) of dietary protein in diets were measured as described in Chapter 3.

5.3.5 Trypsin gene expression

Trypsin gene expression was studied using 2 steps RT-PCR as described in Chapter 3.

5.3.6 Statistical Analysis

Growth performance, survival rate, feed utilization efficiency, protein digestibility and trypsin gene expression data were analyzed using analysis of variance to determine if significant differences exist among treatment means. The Tukey's HSD test was used to determine significant differences between treatments. A 5% error rate for significance was used for analyses. Linear regression analysis of HE substitution levels against survival rate, PER and PPV was conduct including regression of in-vivo protein digestibility against PER and PPV.

5.4 Results

5.4.1 Amino acid composition of experimental diets

Amino acid composition of soybean meal inclusion diets differed from the reference diet with a higher total essential amino acid and also EAA/NEAA (Table 14). Arginine, isoleucine, leucine, phenylalanine, threonine, tryptophan, valine, and aspartic acid of soybean meal inclusion diets were higher whereas histidine, lysine, methionine, alanine, cystine, glutamic acid, glycine, proline, serine, and tyrosine were lower in comparison with the reference diet. Considering amino acid composition among hemoglobin inclusion diets similarity except lysine increased with increasing hemoglobin replacement for fishmeal.

5.4.2 Survival rate

Shrimp receiving the reference diet containing only fishmeal as a main protein source gave the best survival rate ($p<0.05$) compared to 20.53% HE+aa diet fed shrimp followed by the shrimp fed the control diet with fishmeal and soybean meal at the proportion of 60:40

without hemoglobin inclusion (Table 15). Hemoglobin inclusion diet fed shrimp had a growth reduction associated with hemoglobin levels, although 19.8% hemoglobin inclusion with amino acid supplemented diet (19.80% HE+aa) fed shrimp showed survival rate improvement but was not equivalent to that of the control diet. However, the improvement was not found in 26.53% hemoglobin inclusion plus amino acid diet (26.53% HE+aa) fed shrimp and being the lowest survival rate in the trial. Regression of HE levels against survival rate showed $R^2 = 0.39$.

5.4.3 Growth and feed utilization

Final weights of shrimp (Table 15) ranged from 10.54 to 7.09 g/shrimp. Weight gain ranged from 9.51 to 5.71 g/shrimp. SGR ranged from 3.62 to 2.92 %/day. Shrimp fed the reference diet had significantly ($p<0.05$) the highest final weight, weight gain, and SGR, followed by the control diet. Growth rates of shrimp fed hemoglobin inclusion diets were inferior, even with amino acid supplementation.

Feed intake (Table 16) ranged from 9.83 to 10.50 g/shrimp without significant difference among treatments. FCR ranged from 1.11 to 1.78 which was significantly ($p<0.05$) the lowest in the reference diet fed group followed by the control diet and increased with increasing hemoglobin powder inclusion, regardless of amino acid supplementation and that of 26.53% HE+aa diet was significantly the highest. PER ranged from 1.94-1.27, while PPV ranged from 32.04-18.64%. PER and PPV of reference diet were significantly ($p<0.05$) the highest, followed by control diet, while others showed a declining trend with respect to the HE substituted for fishmeal regardless of amino acid supplement, and both of 25.53% HE+aa diet were significantly the lowest and that of reference and control diet was also significantly different. Regression of HE levels against PER and PPV showed $R^2 = 0.54$ and 0.50, respectively.

Proximate composition of final shrimp (Table 17) was significantly different ($p<0.05$) among treatments.

5.4.4 Protein digestibility

Amino group liberated in *in-vitro* protein digestibility (Table 18) ranged from 1.18 to $1.05 * 10^{-7}$ mole ala/200 μL sample. *In-vivo* protein digestibility coefficients ranged from

80.63-85.20%. *In-vitro* and *in-vivo* digestibility coefficients reflected each other for the reference diet which had the highest digestibility, whereas fluctuation of digestibility was observed in other diets. However, the differences among treatments were not statistically significant ($p>0.05$). The regression analysis of *in-vivo* digestibility against PER and PPV showed a non-significantly positive relationship (Figs. 6 and 7).

5.4.5 Trypsin gene expression

The expression of trypsin gene fluctuated among treatments (Fig. 8). However, the trypsin gene expression of 6.73% HE diet was non-significantly the lowest ($p>0.05$) while 13.52% HE diet was the highest compared to other treatments.

5.5 Discussion

Shrimp fed diets containing combination of fishmeal, soybean meal and HE using basal diet having ratio of fishmeal to soybean meal at 60:40 and substituted fishmeal with varying hemoglobin levels either with or without amino acid supplementation showed impaired growth in response to HE replacement regardless of amino acid supplementation. The impaired growth of shrimp fed HE substituted diets might be due to the ineffective protein utilization. Shrimp in all treatments had similar feed intake but the different digestibility of ingested food leading to the varying feed utilization efficiency. The results showed tendency of inverse relationship of apparent crude protein digestibility and FCR. With the similar amount of feed intake, shrimp fed the reference diet had the highest digestibility and the lowest FCR. Shrimp fed the 26.53% HE+aa, in contrast, had the lowest apparent crude protein digestibility and the highest FCR. Apparent crude protein digestibility is positively related with PER and PPV, the more digestible protein the higher PER and PPV as shown in Figures 6 and 7. The reference diet had the best PER and PPV followed by control diet whereas the others had lowering PER and PPV depending on hemoglobin inclusion. The 19.80% HE+aa diet had better PER and PPV than 19.80% HE diet.

Moreover, the impaired growth performance of shrimp fed the HE substitution diets might not be the effect of protein digestibility of the diets, but it might be from antagonism between lysine and arginine. There was evidence of increasing lysine content with increasing levels of HE contrast to a lowering arginine which may have resulted in imbalanced amino acid profile and inability to utilize arginine for muscle growth. Kaushik and Fauconneau (1984) reported some biochemical evidence indicating that some metabolic antagonism may exist between lysine and arginine in rainbow trout, where increasing dietary lysine intake affected plasma arginine and urea levels and ammonia excretion. These changes were due to a decrease in the relative rate of arginine degradation as the level of dietary lysine increased.

The results of this study was contrast with the study by Lim and Dominy (1990) which showed that substitution of marine protein mix (53% anchovy fishmeal, 32% shrimp head meal and 15% squid meal) with defatted SBM at 40% did not impair growth performance of juvenile *L. vannamei* with an average initial weight of 1.02 g/shrimp, however, if considering in the same quantity of fishmeal substitution the results should be similar. *L. vannamei* in this study utilized soybean meal substitute diets less than *P. monodon* reported by Akiyama and FSGP Aquaculture Research (1990) which substitution of animal proteins (fishmeal, squid meal and shrimp meal) with plant proteins (soybean meal and wheat product) at 50.3% with the initial shrimp weight of 3.6 g/shrimp reared in outdoor concrete tank.

Unimpaired feed intake in this study even at the highest level of fishmeal protein substitution (26.53% HE+aa) was probably due to small amount of HE which was only 5.5% of diet. Blood meal has been reported to be unpalatable to channel catfish at 8% (Tucker and Robinson, 1990). The low inclusion level in the present study did not have such effect because the experimental diets also contained fishmeal. Martinez-Llorens *et al*. (2008) revealed that juvenile gilthead sea bream fed diets containing hemoglobin meal at 5% and 10% showed higher feed intake than the control diet. In on-growing size, fish fed 5% hemoglobin meal diet gave the higher feed intake but fish fed 10% hemoglobin meal diet showed lower feed intake compared to control diet.

Blood meal used in combination with other protein sources can improve feed intake. Wang *et al*. (2008) showed that malabar grouper (*Epinephelus malabricus*) fed diets

containing mixed protein (50% poultry by-products meal, 20% meat and bone meal, 20% blood meal and 10% feather meal) substituted for herring meal at 25, 50, 75 and 100% resulted in increasing feed intake and significantly highest at 75% substitution while reduced feed intake was observed at 100% substitution but it was not observed at the substitution less than 50%.

The ability of HE substituted for fishmeal in this study was lower than those in Japanese eel (Lee and Bai, 1997a), Nile tilapia (Lee and Bai, 1997b) and gilthead sea beam (Martinez-Lliorens *et al*., 2008) which used single protein, whereas the recent study used a combination protein of fishmeal and soybean meal. *L. vannamei* fed 6.73% HE diet (combination of three protein sources of FM, SBM and HE) in this study showed lower ability of protein utilization compared with the study of Satoh *et al*. (2003) in rainbow trout using combination four protein sources (FM, SBM, corn gluten meal and blood meal at a ratio of 20:20:25:5) which was not different from the commercial control diet. The difference was caused by the varying ability to utilize hemoglobin of the animals and also imbalanced amino acid profile including EAA/NEAA balance of diets due to essential amino acids containing in mixed ingredients cannot compensate for the limited amino acids.

Wilson (2002) reviewed that shrimp and fish do not have a true crude protein requirement but have a requirement for a well-balanced mixture of essential and non-essential amino acid. This study supported view of Wilson (2002) that reference diet using fishmeal which was a well balanced amino acid protein source gave significantly the best growth compared to others. The other diets which was replaced 40% of fishmeal with soybean meal gave adverse effect on growth could be due to an amino acid deficiency and also essential and non essential amino acid imbalance in soybean meal. A higher impaired growth with increasing hemoglobin levels probably was due to elevated amino acid imbalance caused by the limiting amino acid in hemoglobin. Amino acid supplementation in 19.80% HE+aa and 26.53% HE+aa diets equivalent to that of the control diet did not improve growth perhaps because of amino acid loss prior to intake by shrimp by leaching of uncoated crystal amino acid and also by long period submerging in water before feeding by shrimp compared with rapid feeding in fish. Lim (1993) reported supplemented essential amino acid loss of 26.5% of total amino acid within 1 h of submerging. The loss is amplified by the eating behavior of shrimp which externally masticate their diets

while holding with appendage slowly resulting in high loss, unlike the feeding behavior of many species of fish which catch diet as a whole pellet. In contrast, Huai *et al*. (2009) reported that amino acid supplementation to compensate for amino acid shortage from replacing fishmeal with soybean meal could improve growth in *L. vannamei*.

5.6 Conclusion

High levels of hemoglobin substituted for fishmeal in combination protein of fishmeal, soybean meal and HE resulted in adverse effect on growth performance through amino acid imbalance and protein digestibility. Supplementation of limited amino acid with crystalline amino acids was not effective for growth improvement. Trypsin gene expression did not respond to hemoglobin inclusion in diets. Hemoglobin substituted for fishmeal in three combination protein sources at 6.73% was a reasonable level considering final weight and FCR. In order to diminish expensive fishery protein usage in shrimp diet, protein sparing using lipid should be investigated.

Table 14 Amino acid composition of experimental diets (% of protein)

Amino acid	Experimental diets						
	Reference*	Control	6.73% HE	13.52% HE**	19.80% HE	19.80% HE+aa***	26.53% HE+aa
Arginine	5.46	6.17	6.41	6.28	6.16	6.16	5.55
Histidine	4.19	3.21	3.36	3.40	3.43	3.43	3.34
Isoleucine	1.35	2.67	2.45	2.64	2.81	2.92	2.80
Leucine	5.69	7.36	7.96	8.04	8.11	8.11	7.68
Lysine	6.05	5.91	6.00	6.12	6.23	6.23	6.40
Methionine	2.43	2.16	2.15	2.17	2.18	2.21	2.15
Phenylalanine	4.00	4.68	4.87	4.97	5.06	5.06	4.91
Threonine	3.30	4.43	4.22	4.33	4.43	4.43	4.52
Tryptophan	0.70	0.98	0.80	0.90	0.99	0.99	0.92
Valine	2.01	3.21	3.11	3.58	4.02	4.02	3.73
Alanine	7.58	4.56	4.62	4.69	4.76	4.76	6.53
Aspartic acid	9.45	10.43	10.49	10.31	10.14	10.14	10.26
Cystine	1.09	0.99	0.78	0.80	0.82	0.82	0.87
Glutamic acid	22.95	22.01	21.47	20.98	20.53	20.53	20.34
Glycine	7.67	5.48	5.64	5.35	5.08	5.08	4.93
Proline	7.01	6.82	6.63	6.55	6.48	6.48	6.48
Serine	5.04	5.03	5.06	4.99	4.93	4.93	4.99
Tyrosine	4.02	3.91	3.97	3.90	3.84	3.84	3.61
Essential amino acids	35.18	40.77	41.33	42.42	43.43	43.57	41.99
Non essential amino acids	64.82	59.23	58.67	57.58	56.57	56.57	58.01
EAA/NEAA	0.54	0.69	0.70	0.74	0.77	0.77	0.72

* cited from previous experiment

** by calculation

*** by calculation

Table 15 Growth of *L. vannamei* fed diets with hemoglobin powder (HE) substituted for fishmeal in combination protein sources over 8 week feeding trial

Experimental diets	**Final weight (g/shrimp)**	**Weight gain (g/shrimp)**	**SGR[2] (%/day)**	**Survival rate (%)**
Reference	10.54 ± 0.24^{a1}	9.51 ± 0.21^{a}	3.62 ± 0.03^{a}	86.25 ± 4.79^{a}
Control	8.70 ± 0.68^{b}	7.30 ± 0.67^{b}	3.26 ± 0.13^{b}	77.50 ± 11.90^{ab}
6.73% HE	8.47 ± 0.70^{b}	7.05 ± 0.69^{bc}	3.19 ± 0.14^{bc}	70.00 ± 10.00^{ab}
13.52% HE	7.38 ± 0.41^{bc}	6.25 ± 0.38^{bc}	3.03 ± 0.10^{bc}	68.75 ± 6.29^{ab}
19.80% HE	7.59 ± 0.43^{bc}	6.21 ± 0.41^{bc}	3.05 ± 0.09^{bc}	68.75 ± 10.31^{ab}
19.80% HE + aa	7.46 ± 0.49^{bc}	6.08 ± 0.50^{bc}	3.00 ± 0.13^{bc}	72.50 ± 5.00^{ab}
26.53% HE + aa	7.09 ± 0.88^{c}	5.71 ± 0.88^{c}	2.92 ± 0.24^{c}	60.00 ± 7.07^{b}

[1] Means in a column with the same superscript are not statistically different ($p>0.05$, $n=4$)

[2] Specific growth rate = $(\ln W_2 - \ln W_1)/(T_2-T_1)*100$, W_1 = initial weight, W_2 = final weight, T_2-T_1 = cultured period (days)

Table 16 Feed utilization efficiency of *L. vannamei* fed diets with hemoglobin powder (HE) substituted for fishmeal in combination protein sources over 8 week feeding trial

Experimental diets	Feed intake[2] (g/shrimp)	FCR[3]	PER[4]	PPV[5] (%)
Reference	10.18±0.11	1.11±0.04[c1]	1.94±0.06[a]	32.04+1.26[a]
Control	10.12±0.92	1.40±0.21[bc]	1.59±0.23[b]	24.45+4.40[b]
6.73% HE	10.29±0.81	1.46±0.05[ab]	1.44±0.11[bc]	22.94+3.00[b]
13.52% HE	9.83±0.30	1.58±0.10[ab]	1.39±0.09[bc]	22.34+2.74[b]
19.80% HE	10.50±0.25	1.69±0.10[ab]	1.26±0.07[c]	20.21+1.31[b]
19.80% HE + aa	9.94±0.52	1.65±0.17[ab]	1.38±0.14[bc]	22.50+2.17[b]
26.53% HE + aa	9.99±0.41	1.78±0.25[a]	1.27±0.17[c]	18.64+2.27[b]

[1] Means in a column with the same superscript are not statistically different (p>0.05, n=4)

[2] The reported feed intake was corrected for leaching loss

[3] Feed conversion ratio = feed intake (g)/weight gain (g)

[4] Protein efficiency ratio = weight gain (g)/protein intake (g)

[5] Productive protein value = (protein gain (g)/protein intake (g)) * 100

Table 17 Proximate composition (%) of *L. vannamei* fed diets containing various levels of hemoglobin powder (HE) accompany with amino acid supplementation for 8 weeks (dry matter basis)

Composition	Experimental diets						
	Reference	Control	6.73% HE	13.52% HE	19.80% HE	19.80% HE + aa	26.53%HE + aa
Moisture	75.51±0.20[b1]	76.77±0.25[a]	76.34±0.78[ab]	76.97±0.45[a]	76.63±0.25[a]	76.65±0.27[a]	76.58±0.24[a]
Crude protein	72.51±1.15	72.72±0.07	73.17±1.92	73.07±1.10	73.19±1.40	72.81±0.10	71.42±1.52
Crude fat	3.97±0.99	3.79±0.22	3.51±0.06	3.45±0.86	2.85±0.09	2.56±0.11	2.63±0.53
Ash	12.02±0.40	12.87±0.11	11.96±0.75	11.84±1.32	12.05±0.45	11.97±0.07	12.03±0.48

[1] Means in a row with the same superscript are not statistically different (p>0.05, n=4)

Table 18 *In-vitro* and *in-vivo* protein digestibility of shrimp fed diets containing varying hemoglobin powder (HE) levels substituted for fishmeal

Experimental diets	**AG liberated by experimental feed induced enzyme (10^{-7} mole ala/ 200 µL sample)**	***In-vivo* digestibility (%)**	**Feces (g wet weight/ shrimp/29 days)**
Reference	1.59±0.41	85.20±1.02	2.24±0.50 [b]
Control	1.18±0.08	81.98±1.12	2.51±0.33 [ab]
6.73 % HE	1.40±0.18	81.87±0.71	2.86±0.14 [a]
13.52 % HE	1.32±0.54	82.65±1.31	2.82±0.12 [ab]
19.80 %HE	1.28±0.58	83.67±1.99	2.47±0.41 [ab]
19.80 % HE+ aa	1.50±0.81	82.55±0.59	2.66±0.43 [ab]
26.53 % HE + aa	1.05±0.87	80.63±1.17	2.58±0.23 [ab]

[1] Means in a column with the same superscript are not statistically different (p>0.05, n=3)

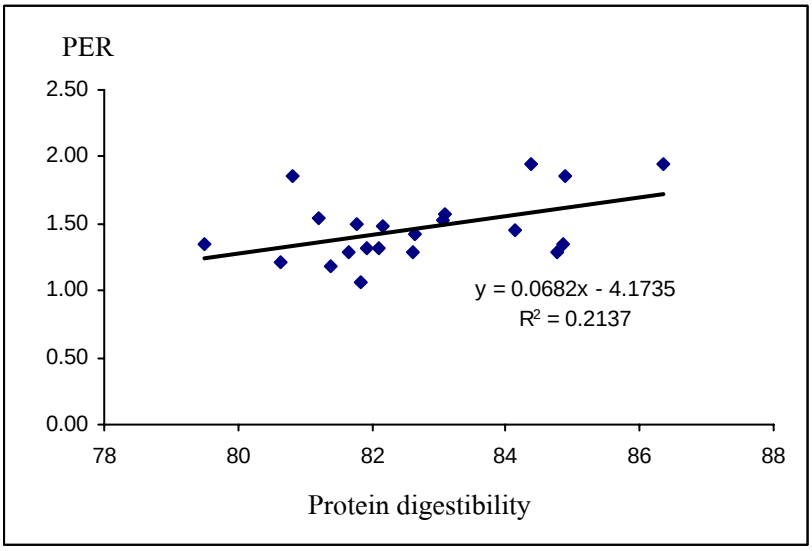

Figure 6 Regression between PER and *in-vivo* protein digestibility of shrimp fed combination protein sources (fishmeal, soybean meal and hemoglobin powder)

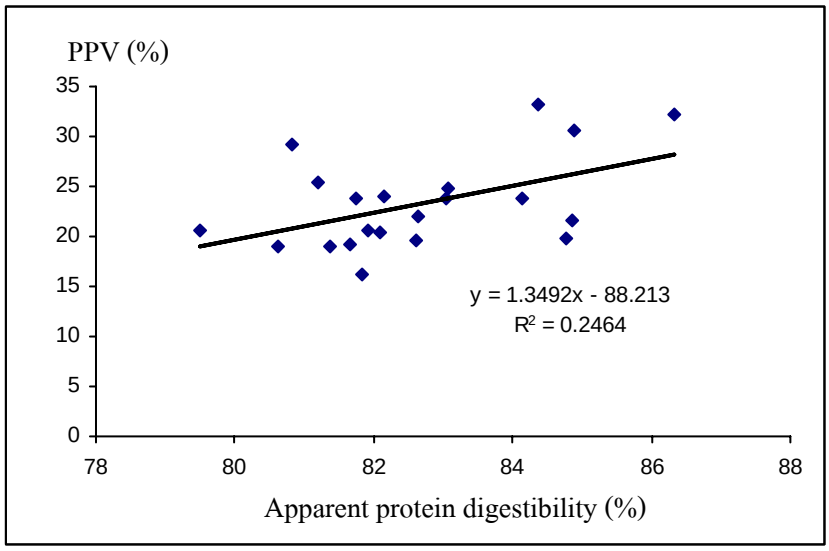

Figure 7 Regression between PPV and *in-vivo* protein digestibility of shrimp fed combination protein sources (fishmeal, soybean meal and hemoglobin powder)

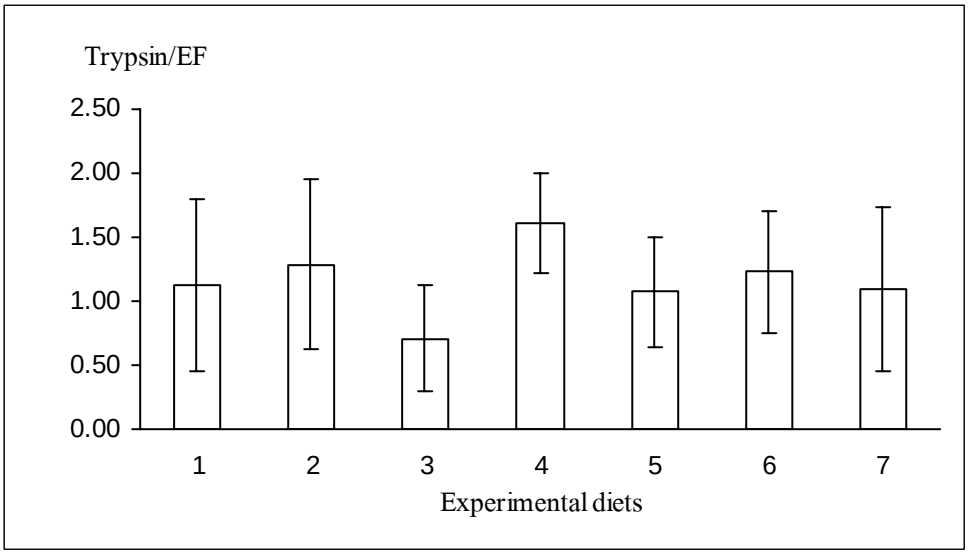

Figure 8 Trypsin gene expression of shrimp fed varying diets, means from 3 replicates (1 = Reference, 2 = Control, 3 = 6.73% HE, 4 = 13.52% HE, 5 = 19.80% HE, 6 = 19.80% HE+aa, 7 = 26.53% HE+aa)

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CHAPTER 6

Protein Sparing Effect and Lipid Quality on Growth Performance, Protein Digestibility and Trypsin Gene Expression in Pacific White Shrimp (*Litopenaeus vannamei*)

6.1 Abstract

An eight week feeding trial was carried out to investigate the protein sparing effect and lipid quality on growth performance, apparent crude protein digestibility and trypsin gene expression of white shrimp. Eight isoenergetic diets were formulated using a factorial design with 3 factors: oil sources (fish oil and soybean oil), lipid levels (8% and 12%) and protein levels (40% and 35%) so that the trial consisted of 8% fish oil with 40% protein (8FO40P), 8% fish oil with 35% protein (8FO35P), 12% fish oil with 40% protein (12FO40P), 12% fish oil with 35% protein (12FO35P), 8% soybean oil with 40% protein (8SO40P), 8% soybean oil 35% protein (8SO35P), 12% soybean oil with 40% protein (12SO40P) and 12% soybean oil 35% protein (12SO35P). At the end of trial greater growth performance was found for soybean oil diet fed shrimp than those fed fish oil diets at the same nutritional composition. Shrimp fed 8% lipid gave good growth whereas those fed with 12% lipid diets resulted in reduced growth irrespective of oil sources and fish oil diets at 12% gave the lowest growth. FCR increased with decreasing protein levels associated with increasing lipid levels. PER and PPV exhibited a trend similar to growth responses which decreased with decreasing protein levels and increasing lipid levels to the lowest PER and PPV in shrimp fed 12% fish oil diets. Shrimp fed soybean oil diets showed higher protein digestibility but decreased with increasing dietary lipid levels. Trypsin gene expression was higher in shrimp fed the 35% protein diets. Protein sparing effects of lipid was not shown but those of carbohydrate could be observed between 8FO40P and 8FO35P diets.

6.2 Introduction

Dietary lipids play an important role as a source of energy for growth and as carriers for fat soluble vitamins. Fish oil contains high quantity of n-3 highly unsaturated fatty

acids and essential fatty acids necessary for marine fish (Sargent and Tacon, 1999). They also serve as a functional element for biological structures, maintaining metabolism and enhance diet palatability. The demand for fish oils in aquafeeds has dramatically increased in the last decade (Barlow, 2000) and has placed unsustainable pressure on fish oil resource (Tacon, 2004). Thus, the partial replacement of fish oils with vegetable oils in artificial feeds has gained increasing interest from aquaculturists (Caddy, 1999; Valdimarsson and James, 2001).

In the past decade, research on dietary lipid in various species often focused on total lipid requirement (Lim *et al*., 1997), essential fatty acids on growth (Gonzalez-Felix *et al*., 2002; Kumaraguru-vasagam *et al.*, 2005) and also vegetable oil substituted for fish oil including lipid quality related to health and flesh quality which affect consumers (Caballero *et al*., 2002; Bransden *et al*., 2003). Total replacement of fish oil with vegetable oil in diets (45% protein and 22% lipid) fed to gilthead sea beam (*Sparus aurata*) resulted in adverse effect on growth and immune responses, whereas the diets with mixed vegetable oil had no significant effect (Montero *et al*., 2008). Moreover, lipid can spare protein in sea beam and European sea bass using 46% protein and 17% lipid diet (Company *et al*., 1999). Protein sparing effect of lipid was also reported in juvenile rockfish fed diet containing 42% protein and 14% lipid (Lee *et al*., 2002).

This study was carried out to reduce fish oil use in shrimp feed through protein sparing effects and finding proper lipid level and sources on growth performance, *in-vitro* and *in-vivo* protein digestibility and trypsin gene expression.

6.3 Materials and Methods

6.3.1 Experimental design

The 2*2*2 factorial experiment in completely randomized design was employed to test two lipid sources (fish oil, FO and soybean oil, SO) at two lipid levels (8% and 12%) and two dietary protein levels (35% and 40%). Pacific white shrimp were cultured in four replicated glass aquaria for each treatment for eight weeks.

6.3.2 Experimental diets and leaching tests

Eight diets were prepared using a combination of fishmeal, hemoglobin powder and soybean meal at a ratio of 56:4:40 as protein sources. Energy content of the diets was adjusted (15.13-15.72 kJ/g diet) using rice and wheat flour.

Fishmeal used in this study was a premium grade (67.52% protein) purchased from fishmeal plant (Pattani Fishmeal Industry Co. Ltd.) in Pattani province, Thailand. Hemoglobin powder (86.05% protein) and soybean meal were donated by a feed company (Intege Feed Co. Ltd). Diet preparation process is the same as described in Chapter 3. The proximate composition of ingredients and experimental diets was determined (AOAC, 1995). The fatty acid profile was determined by gas chromatography (AOAC, 1995).

Diet leaching test was performed using three replicates according to the method modified from Aquacop (1978) and Cruz-Suarez *et al*. (2001) as described in Chapter 3.

6.3.3 Growth trial

Shrimp, culture and feeding

Juvenile *L. vannamei* shrimp were nursed at Aquatic Science Research Station, Satun province, Thailand until used. The shrimp were stocked into 32 glass aquaria (45*45*115 cm) containing 200 L of natural seawater (temperature 26-30 $^{\circ}$C, salinity 10-12 ppt) with 70% water exchange at 7.00 am daily and acclimatized to the experimental conditions for one week. Twenty shrimp with individual initial weight of 1.71$\pm$0.03 g were then selected and randomly distributed into each of 32 glass aquaria and fed with experimental diets. Feeding was done by hand to satiation determined by slow or no response to the diet, 4 times daily at 8.00 am, 12.00 am, 5.00 pm and 10.00 pm for 8 weeks. Uneaten feed was collected for feed intake correction.

Sampling

At the end of the feeding period, ten shrimp from each aquarium were sampled. Six shrimp were used for proximate composition analysis. Two shrimp were decapitated and the hepatopancreas were fixed in TRIzol reagent and kept at -80 $^{\circ}$C until use for trypsin gene expression analysis. Another two shrimp were decapitated then hepatopancreas were taken for

Table 19 Composition (g/100 g), proximate composition (as fed basis) and leaching loss (dry matter basis) of experimental diets

Ingredients	Experimental diets							
	8FO40P	**8FO35P**	**12FO40P**	**12FO35P**	**8SO40P**	**8SO35P**	**12SO40P**	**12SO35P**
Fishmeal (67.52% protein)	23.50	18.80	23.90	19.10	23.50	18.80	23.90	19.10
Hemoglobin meal (86.05% protein)	1.32	1.05	1.34	1.08	1.32	1.05	1.34	1.08
Soybean meal (41.46% protein)	27.40	21.90	27.80	22.30	27.40	21.90	27.80	22.30
Squid meal	8	8	8	8	8	8	8	8
Wheat flour	15	15	17	17	15	15	17	17
Rice flour	9.35	18.70	-	9.50	9.35	18.70	-	9.50
Wheat gluten	5	5	5	5	5	5	5	5
Lecithin	2	2	2	2	2	2	2	2
Tuna fish oil	2.6	3.1	6.6	7.05	-	-	-	-
Soybean oil	-	-	-	-	2.6	3.1	6.6	7.05
Vitamin mix	2	2	2	2	2	2	2	2
Vitamin C	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Mineral mix	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Calcium phosphate	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
BHT	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
CMC	2	2	2	2	2	2	2	2
Cellulose	-	0.62	2.53	3.14	-	0.62	2.53	3.14
98% Chromic oxide	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Proximate composition (as fed basis) and leaching loss (dry matter basis) of experimental diets								
Protein	45.84	40.26	44.64	40.98	45.41	40.32	45.09	39.80
Lipid	8.56	8.31	12.30	12.45	8.07	7.87	11.04	11.45
Moisture	4.98	4.75	4.02	4.02	4.35	4.37	3.88	4.25
Leaching loss (%)	17.08	15.30	17.53	16.17	15.37	14.81	17.43	16.04

[1] Vitamin mix (g/kg vitamin mix): thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0%, biotin 0.05, folic acid 0.18, cyanocobalamine 0.002, chloline chloride 100, inositol 5.0, menadione 2.0, ratinol acetate (20,000 IU/g) 5.0, cholecalciferol (400,000 IU/g) 0.002, DL-alpha-tocopheryl acetate (250 IU/g), wheat flour 865.266

[2] Mineral mix (g/100 g mineral mix): cobalt chloride 0.004, cupric sulfate pentahydrate 0.250, ferrous sulfate 4.0, magnesium heptahydrate 28.398, manganous sulfate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, wheat flour 53.428

enzyme extraction, then pooled from each replicated aquarium and used for *in-vitro* protein digestibility study. Feces were collected throughout the culture period for *in-vivo* protein digestibility investigated.

6.3.4 *In-vitro* protein digestibility

In-vitro protein digestibility coefficient of the experimental diets was determined using crude enzyme extract from the hepatopancreas from the shrimp in the experiments described above. The crude enzyme extract was prepared and the *in-vitro* protein digestibility study was performed using the method modified from Bassompierre (1997) as described in Chapter 3.

6.3.5 *In-vivo* protein digestibility

Apparent digestibility coefficient (ADC) of dietary protein in diets was measured as described in Chapter 3.

6.3.6 Trypsin gene expression

Trypsin gene expression was studied using 2 steps RT-PCR as described in Chapter 3.

6.3.7 Statistical analysis

Growth performance, survival rate, feed utilization efficiency, protein digestibility and trypsin gene expression data were analyzed using a 3-way analysis of variance to determine if significant differences exist among treatment means. The Tukey's HSD test was used to determine significant differences between treatments. A 5% error rate for significance was used for analyses.

6.4 Results

6.4.1 Fatty acid composition of experimental diets

Palmitic acid (C16:0) was the main fatty acid in all diets (Table 20), with more than 15% of lipid in diets, followed by cis-9,12-octadecadienoic acid (C18:2 n-6), cis-9-octadecanoic acid (C18:1 n-9), cis-4,7,10,13,16,19-docosahexaenoic acid (DHA C22:6 n-3) and stearic acid (C18:0). Fish oil diets had much greater content of DHA, eicosapentaenoic acid (EPA C20:5 n-3), stearic acid, palmitic acid, myristic acid, palmitoic acid, heptadecanoic acid, cis-5,8,11,14-eicosatetraenic acid, DHA and an unidentified peak than vegetable oil diets. In contrast, vegetable oil diets had higher amount of cis-9,12-octadecadienoic acid, cis-9-octadecanoic acid and cis-9,12,15-octadecatrienoic acid than fish oil diets. The n-3/n-6 fatty acid of fish oil diets was higher than vegetable oil diets in all treatments except 12SO40P diet. Although, n-3/n-6 of 12FO diets was higher than 8FO diets whereas that of SO diet was similar at both levels except at 12SO40P diet. Although 12SO40P diet has a similar fatty acid composition as FO diets, it had a numerous cis-11,14,17-eicosatrienoic acid (C20:3 n-3) compared with others.

6.4.2 Survival rate

Survival rate of shrimp of all treatments ranged from 71.25-96.25% (Table 21) without significant difference among treatments. Interaction among tested factors (lipid level, lipid source and protein level) was not found. However, shrimp fed soybean oil diets showed higher survival rate of 98.69% as compared with 85.42% of those fed fish oil diets.

6.4.3 Growth and feed utilization

Shrimp fed the 8SO40P diet gave the highest growth performance (final weight, weight gain, and SGR) followed by shrimp fed 8FO35P, 8FO40P, 12SO40P, 8SO35P, 12SO35P, 12FO40P and 12FO35P diets, respectively (Table 21). The 12% SO diets at both protein levels gave higher shrimp growth than 12% fish oil diets regardless of protein levels.

There is no interaction among factors tested on shrimp growth. Regardless of

lipid sources and levels, the 40% protein diets gave a higher final mean weight than the 35% protein diets (8.98 and 7.63 g/shrimp, respectively). SGR of shrimp fed the 40% protein diet was 2.78 whereas that of 35% protein diets was 2.65. Weight gain and SGR were separately affected by lipid sources, lipid levels and protein levels. The SO diets gave higher weight gain than FO diets which was 7.20 and 6.54 g/shrimp, respectively. The 8% lipid diets had better weight gain than 12% lipid diets, which were 7.50 and 6.25 g/shrimp whereas shrimp fed the 40% protein diets had higher weight gain than those fed the 35% protein diets (7.22 and 6.53 g/shrimp, respectively).

Feed intake ranged from 11.22–12.73 g/shrimp (Table 22). Lipid source influenced feed intake; shrimp fed FO diets had higher feed intake than those fed the SO diets (12.06 and 11.53 g/shrimp, respectively). Moreover, interaction among lipid sources, lipid levels and protein levels was also found. Shrimp fed the diet with 35% protein 8% FO had the highest feed intake but those fed the diet with 40% protein 12% SO had the lowest feed intake, while the rest were in a similar range ($p>0.05$).

FCR ranged from 1.44-2.23 (Table 22) which FCR of shrimp fed FO diets (1.90) was higher than that of SO diets (1.63) related to respective treatments. Lipid levels also affected FCR with 12% lipid diets gave higher FCR (1.91) than 8% lipid diets (1.62). Protein levels also influenced FCR, with shrimp fed the 35% protein diets (1.88) had higher FCR than those fed 40% protein diets (1.65) but interaction among factors was not found.

PER and PPV had similar tendencies ranging from 1.12-1.58 and 17.12-25.27%, respectively. Sources and levels of lipid affected both PER and PPV. PER of shrimp fed SO diet was 1.46, whereas those fed FO diets was 1.28. The 8% lipid diets had higher PER than 12% diets, which were 1.48 and 1.26, respectively. Similarly, PPV of SO diets was higher than FO diets which were 23.00 and 19.96%, respectively. PPV of 8% lipid diets was 23.43% whereas that of 12% diets was 19.53%. The interaction among three factors was not found for PER and PPV.

6.4.4 Protein digestibility

In-vitro digestibility ranged from 0.92-1.91x10^{-7} mole alanine/200 μL sample (Table 29). *In-vitro* digestibility was influenced by lipid sources and had interaction ($p<0.05$) between lipid levels and sources. SO diets had higher *in-vitro* digestibility than FO diets (mean of 1.77 and 1.23x10^{-7} M alanine/200 μL sample, respectively). Interaction between lipid level and sources showed that both 8% and 12% SO diets gave the highest ($p<0.05$) *in-vitro* digestibility.

In-vivo digestibility coefficients ranged from 77.21-81.08%. Lipid and protein levels affected digestibility; the 8% lipid diets gave a higher digestibility (80.44%) than the 12% lipid diets (78.59%). Diets containing 40% protein gave the higher digestibility (80.07%) than the 35% protein diets (78.96%). Interaction between lipid and protein levels was significant ($p<0.05$) in this study which at 8% lipid, 40% protein diets had higher digestibility but at 12% lipid, that of 35% protein diets was higher than 40% protein. Interaction between lipid sources and protein levels was also significant ($p<0.05$) which FO diets had greater digestibility at 35% protein level whereas SO diets had a greater digestibility at 40% protein. Moreover, interaction among lipid sources, lipid levels and protein levels was also significant ($p<0.05$) which protein digestibility of 8 and 12% lipid levels diets was greater at high protein level (40%) except that of 12% fish oil inclusion diets which was greater at 35% protein.

6.4.5 Trypsin gene expression

Trypsin gene expression was similar in all treatments. However, with considering average gene expression seem at 35% protein diets giving the higher gene expression that 40% protein diets except at the 12% SO diets due to particularly 12SO40P diet which came from one sample with very high score and out of range compared to others. Besides, lipid levels and lipid sources had no effect on trypsin gene expression in any treatments.

6.5 Discussion

FO diets had high n-3 fatty acid while SO diets had high n-6 fatty acid in this study, similar to those reported by Berge *et al*. (2009) and related to oil sources. The n-3 fatty acid in diets was from FO and oil contained in fishmeal so fatty acid composition in diets varied depending on the quantity of oil sources. FO diets had dramatically higher DHA and EPA which gave higher n-3/n-6 fatty acid than SO diets.

Any lipid sources containing unique fatty acid composition that different oil sources and levels used in diets resulted in different fatty acid composition. FO and SO including fishmeal (associated with the intrinsic lipid of fishmeal) used in this study in varying levels led to the difference of lipid quality with varying fatty acid composition of eight experimental diets.

Different lipid quality diets gave varying growth performance (final weight, weight gain, SGR) of *L. vannamei*. Shrimp fed SO diets showed better growth than FO diets except 8FO35P diet fed shrimp which was better growth than SO diet fed shrimp. Results from this study contrast with *L. vannamei* which shrimp fed FO diet (35% protein and 8% lipid which 6.5% was supplement with various lipid sources) had the best growth compared to linseed oil, SO, corn oil, stearic oil, coconut oil and safflower oil diets (Lim *et al*., 1997). Montero *et al*. (2008) reported that gilthead sea bream fed FO diet (45% protein and 22% lipid) showed better growth than vegetable oil diet but it was similar with the European sea bass fed FO diet and partial replacement of fish oil diets with vegetable oil (rapeseed, linseed and palm oils) that had a equivalent growth (Mourente and Bell, 2006). Similarly, Fountoulaki *et al*. (2009) reported that low fishmeal diet (46% protein and 20% lipid) with 60% substitution FO with SO fed to gilthead sea bream gave equivalent growth with 100% FO diet but it gave adverse effect by using palm oil. It could be due to *L. vannamei* in the present study having a better ability to utilize SO than FO and the essential fatty acids containing in the fishmeal and soybean oil meet requirement of shrimp.

Results in this trial did not show protein sparing effect of lipid in both FO and SO diets because the higher lipid level of the same protein level did not give the better growth. However, the protein sparing effect of carbohydrate was evident in 8% FO diet that 8FO35P diet

containing wheat flour and rice flour at 33.7% of diet gave better growth than 8FO40P diet which had both flour at 24.35% of diet. An increase of carbohydrate could spare 5% protein and shrimp still had good growth. Carbohydrate sparing of protein was not found in SO diets, possibly because n-3 fatty acids particularly EPA and DHA play a key role in the sparing effect process.

Lower growth of 12% oil diets using either oil source was found in this study. A similar result was reported in juvenile *P. monodon* fed isoenergetic and isonitrogenous diets containing between 4 to 11% mixture of cod liver oil and corn oil which showed no difference in weight gain (Sheen *et al*., 1994). Moreover, 12% FO diets gave lower growth than 12% SO diets and 12FO35P diet gave significantly ($p<0.05$) the lowest growth related with increasing DHA, EPA, palmitoic acid and myristic acid in 12% FO diets (Table 20). Poor growth due to receiving high EPA and DHA naturally containing diets was not reported by other researchers. However, Gonzalez-Felix *et al*. (2002) reported that growth rate of juvenile *L. vannamei* was enhanced by addition of DHA or n-3 HUFA mixture containing 416 mg EPA/g and 237 mg DHA/g at 0.25% of diet whereas a higher dietary inclusion level (0.5%) did not further improve growth.

Moreover, final weight, weight gain and specific growth rate decreased with increasing lipid levels. The growth depression due to a higher dietary lipid levels is reported by others. Zhu *et al*. (2009) reported that at the same protein level *L. vannamei* fed 10% lipid diet had a slightly lower growth than 8% level at rearing condition of 2 ppt seawater. Morais *et al*. (2005) reported that high lipid diet with a lower dietary protein/lipid ratio affected fatty acid absorption efficiency in reduction in Senegalese sole (*Solea senegalensis* Kaup 1858) larvae. Poor growth performance of shrimp fed 12FO40P and 12FO35P diet possibly due to bioenergetic aspect. Considering feed intake of shrimp, it can be assumed that shrimp's growth was not only depended on feed intake but also nutrient digestibility. Besides, other causes such as lipid efficiency utilization and energy digestibility which was not investigated are associated with growth depression because fatty acid absorption could be obstructed in the mentioned diets as in Senegalese sole (Morais *et al*., 2005). Even shrimp fed those diets containing the highest lipid level and assumed that they could obtain the equal energy but they might get the lesser energy due to lower digestibility. Similar results were reported by Glencross *et al.* (2002) in *P. monodon* which showed high lipid digestibility of diets containing 4.5, 7.5 and 10.5% lipid, but the

digestibility of the 13.5% lipid diet was lower. Digestibility of total lipids was unaffected by fatty acid composition, except when the level of dietary polyunsaturated fatty acid was 1.7% of the diet and total lipid content was greater than 4.5% (Glencross *et al.*, 2002).

Feed utilization in this study was affected by lipid quality but feed intake was influenced by interaction of lipid level, lipid source and protein level. FO diets gave a higher feed intake than SO diets due to the palatability of FO. Although there were slight differences among treatments, a close range of feed intake indicated that all diets were palatable. Similar palatability among fish oil, sunflower oil, palm oil and rapseed oil was also found for gilthead sea bream (Fountoulaki *et al*., 2009) whereas rapeseed oil was slightly more palatable than fish oil for Atlantic salmon (Bransden *et al*., 2003). FO diets gave higher FCR than SO diets, corresponding with feed intake but all ingested protein cannot be converted into muscle growth at the same level of those treatments. Lipid quality affected FCR, PER, PPV and growth. Interaction among lipid level, lipid source and protein level was not significant for FCR, PER and PPV. However, FCR increased with increasing lipid levels and decreased with increasing protein levels. In contrast, PER and PPV decreased with increasing lipid levels and increased with increasing protein level except for 8FO35P diet.

In-vitro and *in-vivo* protein apparent digestibility coefficients in this study did not reflect each other as observed in the previous experiments (Experiment 1, 2 and 3) but they showed a trend of decreasing with respect to the decreasing protein levels. The trypsin gene expression, in contrast, seemingly was greater at 35% protein than those of 40% protein diet fed shrimp. The inverse relationship of trypsin gene expression to dietary protein level might be due to mechanisms of enzyme regulation by shrimp to compensate the different substrate concentration of different protein levels in diets by increasing amount of enzyme to meet releasing amino acid products velocity and then it resulted in variation in trypsin-enzyme regulator of trypsin gene (Velker, 1996). Besides, increasing lipid levels from 8% to 12% were also inhibit protein digestibility in both cases and ultimately affected growth performance, particularly the lowest growth in 12FO35P shrimp fed diet. Improved protein digestibility of vegetable oil diets in this study was similar to digestibility of Atlantic salmon parr (*Salmo salar*

L.) which was significantly improved when soybean oils were used in the feed (Bendiksen *et al.*, 2003).

6.6 Conclusion

Lipid quality affected growth performance and protein digestibility but had no effect on trypsin gene expression. Unique feed formulation for *L. vannamei* using combination of fishmeal:soybean meal at 60:40 and substituted fishmeal with hemoglobin powder at 6.73% should avoid formulate using 12% fish oil. Formulation at 8% lipid using soybean oil with 40% protein gave good growth but using 8% fish oil showed benefit from protein sparing effects due to carbohydrate and could help reduce feed cost by 5% protein saving.

Table 20 Fatty acid profile (%fatty acid) of experimental diets

Fatty acid	Experimental diets							
	8FO40P	**8FO35P**	**12FO40P**	**12FO35P**	**8SO40P**	**8SO35P**	**12SO40P**	**12SO35P**
C10:0	0.10	-	-	-	-	-	-	-
C12:0	-	-	-	-	-	-	0.05	-
C13:0	-	-	-	-	0.10	-	-	0.05
C14:0	2.57	2.33	3.08	3.04	1.11	1.03	0.88	0.62
C15:0	0.76	0.74	0.94	0.90	0.34	0.29	0.25	0.17
C15:1	-	-	-	-	-	-	0.05	-
C16:0	26.27	27.45	26.64	24.54	22.03	21.65	17.99	17.75
C16:1 n-7	2.48	2.34	3.15	3.14	0.94	0.79	0.78	0.53
C17:0	1.28	1.33	1.61	1.46	0.70	0.57	0.52	0.39
C17:1	0.37	0.39	0.52	0.51	0.17	0.12	0.13	0.09
C18:0	6.37	6.82	6.95	5.97	5.74	5.14	5.11	4.85
C18:1 n-9	11.86	12.68	12.40	11.63	15.33	15.92	19.10	19.42
C18:1 n-7	1.64	1.73	1.89	1.69	1.44	1.28	-	1.14
C18:2 n-6	18.57	17.65	12.33	12.01	36.38	38.51	41.97	44.01
C18:3 n-3	2.18	1.77	1.43	1.49	4.02	4.21	4.91	4.76
C18:3 n-6	0.51	0.32	0.36	0.32	0.25	-	-	-
C18:4 n-3	0.43	0.36	0.47	0.56	0.13	0.10	0.13	0.10
C20:0	0.34	0.37	0.36	0.32	0.33	0.31	0.39	0.37
C20:1 n-9	0.45	0.55	0.62	0.59	0.26	0.24	0.24	0.23
C20:2 n-6	0.14	0.16	0.17	0.17	0.09	0.09	0.08	0.06
C20:3 n-6	0.19	0.13	0.14	0.13	0.07	0.07	0.02	0.05
C20:3 n-3	0.12	0.14	0.10	0.11	0.08	0.05	0.04	-
C20:4 n-6	1.34	1.27	1.32	1.41	0.83	0.67	0.50	0.32
C20:4 n-3	0.19	0.19	0.23	0.25	-	-	0.05	-
C20:5 n-3(EPA)	3.12	2.89	3.33	3.61	1.70	1.42	1.21	1.04
C21:0	-	-	-	-	-	-	0.06	-
C21:5 n-3	0.10	0.12	0.11	0.10	0.09	0.08	-	0.07
C22:1 n-9	0.07	0.15	0.11	0.10	-	0.05	-	0.08
C22:1 n-11, n-13	0.38	0.45	0.58	0.56	-	0.11	-	0.09
C22:4 n-6	0.15	0.15	0.15	0.14	0.11	0.05	-	0.05
C22:5 n-3	0.60	0.60	0.62	0.66	0.36	0.27	0.15	0.17
C22:5 n-6	0.98	1.03	1.13	1.17	0.49	0.39	0.29	0.20
C22:6 n-3(DHA)	12.96	12.55	14.88	16.00	5.66	4.64	3.39	2.32
C23:0	0.11	0.08	0.10	0.12	-	-	-	-
C24:0	0.26	0.29	0.26	0.25	0.23	0.21	0.22	0.18
Unidentified peak	3.10	2.98	4.01	7.05	1.03	1.75	1.47	0.90
n-3/n-6	0.90	0.90	1.36	1.48	0.31	0.27	0.23	0.19
n-3 HUFA (% in diet)	0.98	1.39	2.10	2.59	0.65	0.53	0.40	0.42

Table 21 Growth of *L. vannamei* fed diets containing different lipid sources, lipid levels and protein levels over 8 week feeding trial

Experimental diets	**Final weight (g/shrimp)**	**Weight gain (g/shrimp)**	**SGR[2] (%/day)**	**Survival rate (%)**
8FO40P	9.02±0.78^{ab1}	7.32±0.77^{ab}	2.82±0.13^{ab}	80.00±17.80
8FO35P	9.15±1.63^{ab}	7.46±1.64^{ab}	2.84±0.32^{ab}	71.25±25.94
12FO40P	7.76±0.11^{bc}	6.05±0.10^{bc}	2.56±0.01^{bc}	85.00±7.07
12FO35P	7.06±0.60^{c}	5.35±0.57^{c}	2.40±0.11^{c}	85.00±7.07
8SO40P	9.97±0.65^{a}	8.25±0.62^{a}	2.98±0.08^{a}	96.25±4.79
8SO35P	8.67±0.37^{abc}	6.94±0.37^{abc}	2.74±0.07^{abc}	95.00±4.08
12SO40P	8.98±0.65^{abc}	7.26±0.64^{abc}	2.80±0.11^{ab}	92.50±5.00
12SO35P	8.06±0.97^{abc}	6.34±0.98^{abc}	2.61±0.22^{abc}	95.00±7.07
ANOVA				
Lipid levels	0.306	0.000**	0.000**	0.596
Lipid sources	0.071	0.034**	0.041**	0.001**
Protein levels	0.050**	0.026**	0.018**	0.713
Lipid levels*Lipid sources	0.857	0.140	0.095	0.838
Lipid levels*Protein levels	0.421	0.708	0.553	0.394
Lipid sources*Protein levels	0.723	0.165	0.209	0.541
Lipid levels*Lipid sources*Protein levels	0.601	0.309	0.348	0.902

[1] Means in a column with the same superscript are not statistically different ($p>0.05$, $n=4$)

[2] Specific growth rate = $(\ln W_2 - \ln W_1/T_2-T_1)*100$, W_1 = initial weight, W_2 = final weight, T_2-T_1 = cultured period (days)

** Significant at 95% confidence

Table 22 Feed utilization efficiency of *L. vannamei* fed diets containing different lipid sources, lipid levels and protein levels over 8 week feeding trial

Experimental diets	**Feed intake[2] (g/shrimp)**	**FCR[3]**	**PER[4]**	**PPV[5] (%)**
8FO40P	11.51±0.21^{ab1}	1.63±0.21^{bc}	1.42±0.17	22.31±2.13
8FO35P	12.73±1.29^{a}	1.76±0.42^{abc}	1.44±0.46	22.87±6.93
12FO40P	12.15±0.26^{ab}	1.96±0.10^{ab}	1.12±0.03	17.56±0.41
12FO35P	11.87±0.27^{ab}	2.23±0.20^{a}	1.13±0.10	17.12±2.17
8SO40P	11.89±0.23^{ab}	1.44±0.10^{c}	1.58±0.07	25.27±1.50
8SO35P	11.58±0.20^{ab}	1.67±0.09^{bc}	1.46±0.05	23.26±0.17
12SO40P	11.22±0.42^{b}	1.56±0.15^{bc}	1.47±0.15	22.85±2.60
12SO35P	11.41±0.13^{ab}	1.87±0.25^{abc}	1.32±0.18	20.60±2.94
ANOVA				
Lipid levels	0.228	0.001**	0.017**	0.007**
Lipid sources	0.021**	0.002**	0.038**	0.028**
Protein levels	0.354	0.005**	0.462	0.420
Lipid levels*Lipid sources	0.479	0.120	0.261	0.294
Lipid levels*Protein levels	0.254	0.471	0.943	0.811
Lipid sources*Protein levels	0.233	0.652	0.351	0.396
Lipid levels*Lipid sources*Protein levels	0.030**	0.872	0.943	0.879

[1] Means in a column with the same superscript are not statistically different ($p>0.05$, $n=4$)

[2] The reported feed intake was corrected for leaching loss

[3] Feed conversion ratio = feed intake (g)/weight gain (g)

[4] Protein efficiency ratio = weight gain (g)/protein intake (g)

[5] Productive protein value = (protein gain (g)/protein intake (g)) * 100

** Significant at 95% confidence

Table 23 Proximate composition (%) of *L. vannamei* fed diets containing different lipid sources, lipid levels and protein levels over 8 week feeding trial (dry matter basis)

Indicators	**Experimental diets**							
	8FO40P	**8FO35P**	**12FO40P**	**12FO35P**	**8SO40P**	**8SO35P**	**12SO40P**	**12SO35P**
Moisture	76.88±0.39	76.86±0.67	77.12±0.06	77.58±1.45	76.55±0.10	76.65±0.39	77.28±0.45	76.95±1.20
Crude protein	68.09±1.20	69.06±0.23	68.12±0.78	67.08±0.59	68.04±0.73	68.03±1.62	67.96±0.73	67.29±1.24
Crude fat	3.04±0.16	3.43±1.02	2.56±0.40	3.53±0.53	2.93±0.98	3.63±1.33	3.34±1.23	3.78±1.67
Ash	12.73±0.31	12.50±0.67	13.08±0.33	12.81±0.60	12.42±1.25	12.65±0.38	13.25±0.45	12.61±0.31

[1] Means in a row with the same superscript are not statistically different (p>0.05, n=3)

Table 24 *In-vitro* and *in-vivo* protein digestibility of shrimp fed diets containing different lipid sources, lipid levels and protein levels

Experimental diets	**AG liberated by experimental feed induced enzyme (10^{-7} mole ala/ 200 μL sample)**	**Enzyme Activity (Unit/min/mg protein)**	***In-vivo* digestibility (%)**	**Feces (g wet weight/shrimp/ 29 days)**
8FO40P	1.50±0.29	1.07±0.05^{b1}	81.08±1.39^{ab}	2.72±0.21
8FO35P	1.44±0.12	1.06±0.10^{b}	79.23±0.85^{bcd}	2.69±0.74
12FO40P	1.04±0.41	1.12±0.03^{b}	77.21±0.87^{d}	2.55±0.19
12FO35P	0.92±0.29	1.01±0.03^{b}	79.19±0.23^{bcd}	2.55±0.08
8SO40P	1.67±0.08	1.33±0.02^{a}	82.01±0.76^{a}	2.61±0.48
8SO35P	1.73±0.19	1.15±0.06^{b}	79.43±0.34^{bcd}	2.75±0.47
12SO40P	1.91±0.20	1.12±0.02^{b}	79.96±0.84^{abc}	2.70±0.07
12SO35P	1.76±0.31	1.12±0.04^{b}	78.01±0.52^{cd}	2.62±0.48
ANOVA				
Lipid levels	0.205	-	0.000**	-
Lipid sources	0.003**	-	0.057	-
Protein levels	0.621	-	0.004**	-
Lipid levels*Lipid sources	0.041**	-	0.736	-
Lipid levels*Protein levels	0.622	-	0.004**	-
Lipid sources*Protein levels	0.876	-	0.003**	-
Lipid levels*Lipid sources*Protein levels	0.779	-	0.026**	-

[1] Means in a column with the same superscript are not statistically different (p>0.05, n=3)

** Significant at 95% confidence

Figure 9 Trypsin gene expression of *L. vannamei* fed diets containing different lipid sources (fish oil and soybean oil) at two lipid levels (8 and 12%) and protein levels (35 and 40%) (n=3 except *, n=1)

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CHAPTER 7

Conclusions and Recommendation

Experiment 1 clearly demonstrated a relationship between Protein digestibility and fishmeal grade based on criteria according to Thai meal quality standard (The Ministry of Agriculture and Co-operative (1995) and Thai Fishmeal Producer Association (2003) with a higher *in-vivo* protein apparent digestibility in a group of imported FM, premium grade FM (S1) and Premium grade FM (S2) followed by grade 1 FM and grade 2 FM. The key issue about irrelevant assessment technique for Protein digestibility was found with the highest *in-vitro* protein digestibility reverse to the lowest *in-vivo* protein digestibility in the same protein source of grade 2 FM. Growth responses was associated with protein digestibility and amino acid composition particularly EAA/NEAA. As a result, premium grade FM (S1) having a good amino acid balance and the highest apparent crude protein digestibility promoted the highest growth, although, grade 1 FM having a similar amino acid profile but its lower protein digestibility affected the availability of essential amino acid resulting in impaired growth. Trypsin gene expression related to fishmeal quality.

When fishmeal was substituted with hemoglobin powder in Experiment 2, protein digestibility increased with increasing hemoglobin levels. However, replacing fishmeal with hemoglobin powder at high level resulted in depressed growth due to the imbalance amino acid. Trypsin gene expression was not related to protein quality due to hemoglobin replacement for fishmeal in Experiment 2. Hemoglobin powder was unsuccessful to substitute for fishmeal.

Protein digestibility and growth of shrimp fed combination protein sources with and without limiting amino acid supplementation diets in Experiment 3 reduced with increasing hemoglobin inclusion in diets. Trypsin gene expression was not related with protein quality due to hemoglobin replacement for fishmeal in combination protein.

Protein digestibility, growth performance and feed utilization efficiency of shrimp were influenced by lipid levels and sources. Diets containing 8% lipid was better than 12% lipid regardless of oil source and soybean oil was better than fish oil. Moreover, 40% protein was better than 35% protein except at 8% fish oil with 35% protein caused by protein sparing effect from carbohydrate. Protein sparing effect with lipid was not found.

The results from these studies indicate that protein digestibility in juvenile *L. vannamei* varies in response to fishmeal grade, levels of digestible protein source and lipid quality. Moreover, well digested protein cannot support a good growth unless amino acid imbalance is mitigated and a good combination of feed ingredients among protein, lipid and carbohydrate are provided.

Recommendation

To avoid lower growth as found in Experiment 3, soybean meal should be less than 40% which will allow fishmeal substitution with hemoglobin powder at a higher level than 6.73%. The form of amino acid supplementation is crucial for availability to animals. A study on others vegetable oils, such as palm oil which is the main product in southern Thailand, should be done.

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APPENDIX 1

Chemical Analysis

Determination of total amino acid in foods using HPLC (AOAC, 1995)

Principle of analysis

Food containing protein was digested with 6.0 N HCl at 110 °C for 22 huntil obtained hydrolyzed amino acid. The obtained solution was analyzed using specific HPLC (High Performance Liquid Chromatograph) for amino acid with the proper condition, column and mobile phase. Individual amino acid which was passed column was reacted with OPA (o-phthalaldehyde) to form fluorescence and was detected by fluorescence detector. Then the data was passed to computer for computing and the results can be reported in both quantitative and qualitative.

This system can be analyzed seventeen amino acids which consisted of

1. Aspartic acid
2. Threonine
3. Serine
4. Glutamic acid
5. Proline
6. Glycine
7. Alanine
8. Cystine
9. Valine
10. Methionine
11. Isoleucine
12. Leucine
13. Tyrosine
14. Phenylalanine
15. Histidine
16. Lysine
17. Arginine

Amino acids were analyzed and compared with amino acid standard H (PIERCE)

Reagent

1. Hydrochloric acid
2. Sodium hydroxide
3. Ethanol 99.5%
4. Sodium citrate HPLC grade
5. Perchloric acid 60%
6. Boric acid
7. Sodium carbonate
8. Potassium sulphate
9. Brij-35
10. Sodium hypochlorite
11. OPA (o-phthalaldehyde)
12. N-Acetyl-L-Cystein
13. n-carpyric acid

Apparatus and Materials

1. High Performance Liquid Chromatograph for amino acid
2. Four-digits balance
3. Two-digits balance
4. Filter paper (Whatman No. 40, 41)
5. Volumetric flask size 10, 25 and 100 mL
6. Volume pipette size 1, 2, 5 and 10 mL
7. Measuring pipette size 1, 2, 5, 10, 25 mL
8. Beaker size 50, 250, 500, 1000, 2000, 3000 mL
9. Erlenmeyer flask size 250 and 500 mL
10. Cylinder size 10, 50, 100 and 500 mL
11. Vial size 5, 10, 25 mL

12. Hot plate
13. Sand bath (for sample digestion instead of reaction box)
14. Reaction tube set for sample digestion
15. Hot plate with magnetic stirrer
16. Aspirator
17. pH meter
18. Plastic funnel (small size) and pipette ball
19. Squeeze bottles with distilled water
20. Pasteur pipettes
21. Hood
22. Glass and plastic bottles using for substance and mobile phase containing

Analytical conditions

High performance liquid chromatograph amino acid analysis model LC-6A; Shimadzu; Japan

Column : Shim-pack ISO-07/s 1504 Na

(packed with cation exchanger consist of sulphonate syrene divinyl benzene copolymer)

Mobile phase : A = 0.2 N sodium citrate (containing 7% EtOH, pH 3.2)

B = 0.6 N sodium citrate + 0.2 N boric acid, pH 10

C = 0.2 N sodium hydroxide

Flow rate : 0.3 ml/min

Detector :Fluorescence detector

Reaction temp. : 55°C

Reaction reagent

Reaction A = 0.4 mL commercial sodium hypochlorite/ 1 L alkaline buffer

Reaction B = 0.8 g o-phthal aldehyde/ 14 mL EthOH

0.4 g polyoxyethylene lauryl ether (Brij-35)

1.0 n-acetyl-L-cysteine

Add to 1 L alkaline buffer

Flow rate of reaction reagent = 0.3 mL/min Alkaline buffer

0.384 M Sodium carbonate

0.216 M Boric acid

0.108 M Potassium sulphate pH 10

Diluent sample and standard amino acid solution

0.2 N Sodium citrate

1.5% Perchloric acid

0.05% n-caprylic acid

pH 2.2

Sample preparation

Acid hydrolysis method (for amino acid profile)

Wt, 52.3 mg sample in vacuum tube (reaction tube)

↓

Added 3-4 mL of 6 N HCl sealed tube under reduced pressure

↓

Hydrolyzed at 110°C for 22-24 h.

Allowed to cool

↓

Made up volume with diluent sample, filter

↓

Injected 20 µL into HPLC (Amino acid analyzer)

Reference standard: Amino acid standard H (std. mix Asp….Arg except Tryptophan) (Pierce chemical company)

Alkaline hydrolysis

Wt, sample 51.1 mg

↓

Added 2 mL of 4.2 N NaOH

Sealed tube under reduced pressure

↓

Hydrolyzed at 110°C for 16 h

↓

Neutralized with HCl with cooling

↓

Adjusted volume to 10 mL

↓

Decant and filter solution

↓

Injected 25 μL into HPLC (Amino acid analyzer)

Time program amino acid analysis

Time (min)	func	Value (%)
0.01	Zero	
5.0	B conc	0
22.0	”	14
26.0	”	14
26.01	”	60
40.0	”	100
50.0	”	100
50.01	”	0
50.02	RV.C	6
55.0	”	1
80.0	stop	

Calculation

$$\text{Amino acid content} = \frac{\text{MW} * \text{area sample} * \text{DF} * \text{total volume } (\mu\text{L}) * 100}{\text{Area std} * \text{wt. of sample (g)} * 10 * 10^{6}}$$

MW = molecular weight of amino acid

DF = dilution factor

Reference

Shimadzu HPLC amino acid analysis system application data book.; Shimadzu Co., Ltd

APPENDIX 2

Statistical Analysis

Appendix 2.1 ANOVA of experiment 1

		Sum of Squares	df	Mean Square	F	Sig.
Survival	Between Groups	256.000	4	64.000	2.017	0.144
	Within Groups	476.000	15	31.733		
	Total	732.000	19			
Final weight	Between Groups	8.421	4	2.105	9.260	0.001
	Within Groups	3.410	15	0.227		
	Total	11.832	19			
Weight gain	Between Groups	8.435	4	2.109	9.316	0.001
	Within Groups	3.395	15	0.226		
	Total	11.831	19			
SGR	Between Groups	0.421	4	0.105	9.326	0.001
	Within Groups	0.169	15	0.011		
	Total	0.590	19			
Feed intake	Between Groups	10.873	4	2.718	16.774	0.000
	Within Groups	2.431	15	0.162		
	Total	13.304	19			
FCR	Between Groups	0.163	4	0.041	3.931	0.022
	Within Groups	0.155	15	0.010		
	Total	0.318	19			
PER	Between Groups	0.227	4	0.057	4.433	0.015
	Within Groups	0.192	15	0.013		
	Total	0.419	19			
PPV	Between Groups	86.195	4	21.549	5.177	0.008
	Within Groups	62.439	15	4.163		

Appendix 2.1 ANOVA of experiment 1 (continue)

		Sum of Squares	df	Mean Square	F	Sig.
In-vitro protein digestibility	Between Groups	0.308	4	0.077	1.714	0.230
	Within Groups	0.404	9	0.045		
	Total	0.712	13			
In-vivo protein digestibility	Between Groups	112.224	4	28.056	76.859	0.000
	Within Groups	5.476	15	0.365		
	Total	117.700	19			

Appendix 2.2 ANOVA of experiment 2

		Sum of Squares	df	Mean Square	F	Sig.
Survival	Between Groups	470.833	5	94.167	2.511	0.068
	Within Groups	675.000	18	37.500		
	Total	1145.833	23			
Final weight	Between Groups	48.160	5	9.632	92.014	0.000
	Within Groups	1.884	18	0.105		
	Total	50.044	23			
Weight gain	Between Groups	48.137	5	9.627	54.732	0.000
	Within Groups	3.166	18	0.176		
	Total	51.304	23			
SGR	Between Groups	4.515	5	0.903	92.614	0.000
	Within Groups	0.176	18	0.010		
	Total	4.690	23			
Feed intake	Between Groups	11.390	5	2.278	28.722	0.000
	Within Groups	1.428	18	0.079		
	Total	12.818	23			
FCR	Between Groups	5.190	5	1.038	62.742	0.000
	Within Groups	0.298	18	0.017		
	Total	5.488	23			
PER	Between Groups	2.282	5	0.456	44.735	0.000
	Within Groups	0.184	18	0.010		
	Total	2.466	23			
PPV	Between Groups	667.370	5	133.474	25.791	0.000
	Within Groups	51.751	10	5.175		
	Total	719.121	15			
Survival	Between Groups	470.833	5	94.167	2.511	0.068
	Within Groups	675.000	18	37.500		

Appendix 2.2 ANOVA of experiment 2 (continue)

		Sum of Squares	df	Mean Square	F	Sig.
	Total	1145.833	23			
In-vitro protein	Between Groups	0.473			2.846	0.118
digestibility	Within Groups	0.199	6	0.033		
	Total	0.672	11			
In-vivo protein	Between Groups	14.471	5	2.894	1.465	0.272
digestibility	Within Groups	23.703	12	1.975		
	Total	38.174	17			
Gene	Between Groups	0.348	5	0.070	0.439	0.813
	Within Groups	1.903	12	0.159		
	Total	2.251	17			

Appendix 2.3 ANOVA of experiment 3

		Sum of Squares	df	Mean Square	F	Sig.
Survival	Between Groups	1606.713	6	267.785	3.949	0.009
	Within Groups	1356.250	20	67.813		
	Total	2962.963	26			
Final weight	Between Groups	34.390	6	5.732	17.314	0.000
	Within Groups	6.621	20	0.331		
	Total	41.011	26			
Weight gain	Between Groups	32.610	6	5.435	16.806	0.000
	Within Groups	6.468	20	0.323		
	Total	39.077	26			
SGR	Between Groups	1.328	6	0.221	12.131	0.000
	Within Groups	0.365	20	0.018		
	Total	1.692	26			
Feed intake	Between Groups	1.129	6	0.188	0.642	0.696
	Within Groups	6.160	21	0.293		
	Total	7.289	27			
FCR	Between Groups	1.183	6	0.197	8.119	0.000
	Within Groups	0.510	21	0.024		
	Total	1.692	27			
PER	Between Groups	1.339	6	0.223	11.789	0.000
	Within Groups	0.398	21	0.019		
	Total	1.736	27			
PPV		332.056	6	55.343	7.902	0.001
		98.053	14	7.004		
		430.108	20			

Appendix 2.3 ANOVA of experiment 3 (continue)

		Sum of Squares	df	Mean Square	F	Sig.
In-vitro protein digestibility	Between Groups	0.472	6	0.083	0.386	0.872
	Within Groups	2.037	10	0.276		
	Total	2.509	16			
In-vivo protein digestibility	Between Groups	38.121	6	6.353	4.364	0.011
	Within Groups	20.380	14	1.456		
	Total	58.501	20			

Appendix 2.4 ANOVA of Survival of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	719.583[a]	7	102.798	2.202	0.074
Intercept	239538.542	1	239538.542	5130.886	0.000
Lipid levels	13.542	1	13.542	0.290	0.596
Lipid sources	634.696	1	634.696	13.595	0.001
Protein levels	6.490	1	6.490	0.139	0.713
Lipid levels*Lipid sources	2.003	1	2.003	0.043	0.838
Lipid levels*Protein levels	35.337	1	35.337	0.757	0.394
Lipid sources*Protein levels	18.029	1	18.029	0.386	0.541
Lipid levels*Lipid sources*Protein levels	0.721	1	0.721	0.015	0.902
Error	1027.083	22	46.686		
Total	246550.000	30			
Corrected Total	1746.667	29			

a, R Squared = 0.412 (Adjusted R Squared = 0.225)

Appendix 2.5 Estimated marginal means of survival

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		90.729	1.845	86.903	94.556
12%		89.375	1.708	85.832	92.918
Lipid sources					
Fish oil		85.417	1.845	81.590	89.243
Soybean oil		94.688	1.708	91.145	98.230
Protein levels					
35%		89.583	1.778	85.896	93.271
40%		90.521	1.778	86.834	94.208
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	85.833	2.789	80.048	91.618
	Soybean oil	95.625	2.416	90.615	100.635
12%	Fish oil	85.000	2.416	79.990	90.010
	Soybean oil	93.750	2.416	88.740	98.760
Lipid levels*Protein levels					
Lipid levels	Protein levels				
8%	35%	89.167	2.609	83.755	94.578
	40%	92.292	2.609	86.880	97.703
12%	35%	90.000	2.416	84.990	95.010
	40%	88.750	2.416	83.740	93.760

Appendix 2.5 Estimated marginal means of survival (continue)

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Lipid sources*Protein levels						
Lipid sources	Protein levels					
Fish oil	35%		84.167	2.609	78.755	89.578
	40%		86.667	2.609	81.255	92.078
Soybean oil	35%		95.000	2.416	89.990	100.010
	40%		94.375	2.416	89.365	99.385
Lipid levels* Lipid sources* Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	83.333	3.945	75.152	91.514
		40%	88.333	3.945	80.152	96.514
	Soybean oil	35%	95.000	3.416	87.915	102.085
		40%	96.250	3.416	89.165	103.335
12%	Fish oil	35%	85.000	3.416	77.915	92.085
		40%	85.000	3.416	77.915	92.085
	Soybean oil	35%	95.000	3.416	87.915	102.085
		40%	92.500	3.416	85.415	99.585

Appendix 2.6 ANOVA of final weight of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	30.917[a]	7	4.417	1.418	0.248
Intercept	2037.204	1	2037.204	653.973	0.000
Lipid levels	3.421	1	3.421	1.098	0.306
Lipid sources	11.206	1	11.206	3.597	0.071
Protein levels	13.446	1	13.446	4.316	0.050
Lipid levels*Lipid sources	0.103	1	0.103	0.033	0.857
Lipid levels*Protein levels	2.099	1	2.099	0.674	0.421
Lipid sources*Protein levels	0.401	1	0.401	0.129	0.723
Lipid levels*Lipid sources*Protein levels	0.876	1	0.876	0.281	0.601
Error	68.533	22	3.115		
Total	2179.617	30			
Corrected Total	99.449	29			

a, R Squared = 0.311 (Adjusted R Squared = 0.092)

Appendix 2.7 Estimated marginal means of final weight

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		8.645	0.477	7.657	9.633
12%		7.964	0.441	7.049	8.879
Lipid sources					
Fish oil		7.689	0.477	6.700	8.677
Soybean oil		8.921	0.441	8.006	9.836
Protein levels					
35%		7.630	0.459	6.678	8.582
40%		8.979	0.459	8.027	9.932
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	7.970	0.721	6.476	9.464
	Soybean oil	9.320	0.624	8.026	10.614
12%	Fish oil	7.408	0.624	6.113	8.702
	Soybean oil	8.521	0.624	7.227	9.815
Lipid levels*Protein levels					
Lipid levels	Protein levels				
8%	35%	7.704	0.674	6.306	9.102
	40%	9.586	0.674	8.188	10.984
12%	35%	7.556	0.674	6.262	8.850
	40%	8.373	0.674	7.078	9.667

Appendix 2.7 Estimated marginal means of final weight (continue)

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Lipid sources*Protein levels						
Lipid sources	Protein levels					
Fish oil	35%		6.898	0.674	5.500	8.295
	40%		8.480	0.674	7.082	9.878
Soybean oil	35%		8.363	0.674	7.068	9.657
	40%		9.479	0.674	8.185	10.773
Lipid levels* Lipid sources* Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	6.740	1.019	4.627	8.853
		40%	9.200	1.019	7.087	11.313
	Soybean oil	35%	8.668	0.882	6.837	10.498
		40%	9.973	0.882	8.142	11.803
12%	Fish oil	35%	7.055	0.882	5.225	8.885
		40%	7.760	0.882	5.930	9.590
	Soybean oil	35%	8.058	0.882	6.227	9.888
		40%	8.985	0.882	7.155	10.815

Appendix 2.8 ANOVA of weight gain of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	23.568[a]	7	3.367	4.897	0.002
Intercept	1511.675	1	1511.675	2198.487	0.000
Lipid levels	12.375	1	12.375	17.998	0.000
Lipid sources	3.458	1	3.458	5.030	0.034
Protein levels	3.878	1	3.878	5.640	0.026
Lipid levels*Lipid sources	1.602	1	1.602	2.330	0.140
Lipid levels*Protein levels	0.099	1	0.099	0.144	0.708
Lipid sources*Protein levels	1.411	1	1.411	2.052	0.165
Lipid levels*Lipid sources*Protein levels	0.744	1	0.744	1.082	0.309
Error	16.502	24	0.688		
Total	1551.746	32			
Corrected Total	40.071	31			

a, R Squared = 0.588 (Adjusted R Squared = 0.468)

Appendix 2.9 Estimated marginal means of weight gain

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		7.495	0.207	7.067	7.923
12%		6.251	0.207	5.823	6.679
Lipid sources					
Fish oil		6.544	0.207	6.117	6.972
Soybean oil		7.202	0.207	6.774	7.630
Protein levels					
35%		6.525	0.207	6.097	6.953
40%		7.221	0.207	6.793	7.649
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	7.390	0.293	6.785	7.995
	Soybean oil	7.600	0.293	6.995	8.205
12%	Fish oil	5.699	0.293	5.094	6.304
	Soybean oil	6.804	0.293	6.199	7.409
Lipid levels*Protein levels					
Lipid levels	Protein levels				
8%	35%	7.203	0.293	6.597	7.808
	40%	7.788	0.293	7.182	8.393
12%	35%	5.848	0.293	5.242	6.453
	40%	6.655	0.293	6.050	7.260

Appendix 2.9 Estimated marginal means of weight gain (continue)

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Lipid sources*Protein levels						
Lipid sources	Protein levels					
Fish oil	35%		6.406	0.293	5.801	7.011
	40%		6.683	0.293	6.077	7.288
Soybean oil	35%		6.644	0.293	6.039	7.249
	40%		7.760	0.293	7.155	8.365
Lipid levels*Lipid sources*Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	7.460	0.415	6.604	8.316
		40%	7.320	0.415	6.464	8.176
	Soybean oil	35%	6.945	0.415	6.089	7.801
		40%	8.255	0.415	7.399	9.111
12%	Fish oil	35%	5.353	0.415	4.497	6.208
		40%	6.045	0.415	5.189	6.901
	Soybean oil	35%	6.343	0.415	5.487	7.198
		40%	7.265	0.415	6.409	8.121

Appendix 2.10 ANOVA of specific growth rate of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	0.940[a]	7	0.134	5.329	0.001
Intercept	236.205	1	236.205	9369.346	0.000
Lipid levels	0.510	1	0.510	20.232	0.000
Lipid sources	0.118	1	0.118	4.665	0.041
Protein levels	0.162	1	0.162	6.444	0.018
Lipid levels*Lipid sources	0.076	1	0.076	3.017	0.095
Lipid levels*Protein levels	0.009	1	0.009	0.361	0.553
Lipid sources*Protein levels	0.042	1	0.042	1.668	0.209
Lipid levels*Lipid sources*Protein levels	0.023	1	0.023	0.917	0.348
Error	0.605	24	0.025		
Total	237.751	32			
Corrected Total	1.545	31			

a, R Squared = 0.609 (Adjusted R Squared = 0.494)

Appendix 2.11 Estimated marginal means of specific growth rate

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		2.843	0.040	2.761	2.925
12%		2.591	0.040	2.509	2.673
Lipid sources					
Fish oil		2.574	0.040	2.574	2.738
Soybean oil		2.696	0.040	2.696	2.859
Protein levels					
35%		2.646	0.040	2.564	2.728
40%		2.788	0.040	2.706	2.870
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	2.831	0.056	2.715	2.947
	Soybean oil	2.855	0.056	2.739	2.971
12%	Fish oil	2.481	0.056	2.365	2.597
	Soybean oil	2.700	0.056	2.584	2.816
Lipid levels*Protein levels					
Lipid levels	Protein levels				
8%	35%	2.789	0.056	2.673	2.905
	40%	2.898	0.056	2.782	3.013
12%	35%	2.503	0.056	2.387	2.618
	40%	2.679	0.056	2.563	2.795

Appendix 2.11 Estimated marginal means of specific growth rate (continue)

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Lipid sources*Protein levels						
Lipid sources	Protein levels					
Fish oil	35%		2.621	0.056	2.505	2.737
	40%		2.691	0.056	2.575	2.807
Soybean oil	35%		2.670	0.056	2.554	2.786
	40%		2.885	0.056	2.769	3.001
Lipid levels*Lipid sources*Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	2.840	0.079	2.676	3.004
		40%	2.823	0.079	2.659	2.986
	Soybean oil	35%	2.738	0.079	2.574	2.901
		40%	2.973	0.079	2.809	3.136
12%	Fish oil	35%	2.403	0.079	2.239	2.566
		40%	2.560	0.079	2.396	2.724
	Soybean oil	35%	2.603	0.079	2.439	2.766
		40%	2.798	0.079	2.634	2.961

Appendix 2.12 ANOVA of feed intake of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.840[a]	7	0.691	2.599	0.054
Intercept	3338.693	1	3338.693	12550.887	0.000
Lipid levels	0.419	1	0.419	1.574	0.228
Lipid sources	1.744	1	1.744	6.557	0.021
Protein levels	0.242	1	0.242	0.910	0.354
Lipid levels*Lipid sources	0.140	1	0.140	0.525	0.479
Lipid levels*Protein levels	0.373	1	0.373	1.400	0.254
Lipid sources*Protein levels	0.408	1	0.408	1.535	0.233
Lipid levels*Lipid sources*Protein levels	1.515	1	1.515	5.695	0.030
Error	4.256	16	0.266		
Total	3347.789	24			
Corrected Total	9.096	23			

a, R Squared = 0.532 (Adjusted R Squared = 0.327)

Appendix 2.13 Estimated marginal means of feed intake

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		11.927	0.149	11.611	12.242
12%		11.663	0.149	1.347	11.978
Lipid sources					
Fish oil		12.064	0.149	11.749	12.380
Soybean oil		11.525	0.149	1.209	11.841
Protein levels					
35%		11.895	0.149	11.579	12.211
40%		1.694	0.149	11.379	12.010
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	12.120	0.211	11.674	12.566
	Soybean oil	11.733	0.211	11.287	12.180
12%	Fish oil	12.008	0.211	11.562	12.455
	Soybean oil	11.317	0.211	10.870	11.763
Lipid levels*Protein levels					
Lipid levels	Protein levels				
8%	35%	12.152	0.211	11.705	12.598
	40%	11.702	0.211	11.255	12.148
12%	35%	11.638	0.211	11.192	12.085
	40%	11.687	0.211	11.240	12.133

Appendix 2.13 Estimated marginal means of feed intake (continue)

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Lipid sources*Protein levels						
Lipid sources	Protein levels					
Fish oil	35%		12.295	0.211	11.849	12.741
	40%		11.833	0.211	11.387	12.280
Soybean oil	35%		1.495	0.211	11.049	11.941
	40%		11.555	0.211	11.109	12.001
Lipid levels*Lipid sources*Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	12.727	0.298	12.095	13.358
		40%	11.513	0.298	10.882	12.145
	Soybean oil	35%	11.577	0.298	10.945	12.208
		40%	11.890	0.298	11.259	12.521
12%	Fish oil	35%	11.863	0.298	11.232	12.495
		40%	12.153	0.298	11.522	12.785
	Soybean oil	35%	11.413	0.298	10.782	12.045
		40%	11.220	0.298	10.589	11.851

Appendix 2.14 ANOVA of feed conversion ratio of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.799[a]	7	0.257	5.457	0.001
Intercept	99.687	1	99.687	2116.407	0.000
Lipid levels	0.638	1	0.638	13.555	0.001
Lipid sources	0.546	1	0.546	11.592	0.002
Protein levels	0.456	1	0.456	9.681	0.005
Lipid levels*Lipid sources	0.123	1	0.123	2.601	0.120
Lipid levels*Protein levels	0.025	1	0.025	0.537	0.471
Lipid sources*Protein levels	0.010	1	0.010	0.208	0.652
Lipid levels*Lipid sources*Protein levels	0.001	1	0.001	0.027	0.872
Error	1.130	24	0.047		
Total	102.617	32			
Corrected Total	2.930	31			

a, R Squared = 0.614 (Adjusted R Squared = 0.502)

Appendix 2.15 Estimated marginal means of feed conversion ratio

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		1.624	0.054	1.512	1.736
12%		1.906	0.054	1.794	2.018
Lipid sources					
Fish oil		1.896	0.054	1.784	2.008
Soybean oil		1.634	0.054	1.522	1.746
Protein levels					
35%		1.884	0.054	1.772	1.996
40%		1.646	0.054	1.534	1.758
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	1.693	0.077	1.534	1.851
	Soybean oil	1.555	0.077	1.397	1.713
12%	Fish oil	2.099	0.077	1.940	2.257
	Soybean oil	1.714	0.077	1.555	1.872
Lipid levels*Protein levels					
8%	35%	1.715	0.077	1.557	1.873
	40%	1.533	0.077	1.374	1.691
12%	35%	2.054	0.077	1.895	2.212
	40%	1.759	0.077	1.600	1.917
Lipid sources*Protein levels					
Fish oil	35%	1.998	0.077	1.839	2.156
	40%	1.794	0.077	1.635	1.952

Appendix 2.15 Estimated marginal means of feed conversion ratio (continue)

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Soybean oil	35%		1.771	0.077	1.613	1.930
	40%		1.498	0.077	1.339	1.656
Lipid sources*Protein levels						
Fish oil	35%		1.998	0.077	1.839	2.156
	40%		1.794	0.077	1.635	1.952
Soybean oil	35%		1.771	0.077	1.613	1.930
	40%		1.498	0.077	1.339	1.656
Lipid levels*Lipid sources*Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	1.760	0.109	1.536	1.984
		40%	1.625	0.109	1.401	1.849
	Soybean oil	35%	1.670	0.109	1.446	1.894
		40%	1.440	0.109	1.216	1.664
12%	Fish oil	35%	2.235	0.109	2.011	2.459
		40%	1.963	0.109	1.739	2.186
	Soybean oil	35%	1.873	0.109	1.649	2.096
		40%	1.555	0.109	1.331	1.779

Appendix 2.16 ANOVA of protein efficiency ratio of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	0.591[a]	7	0.084	2.160	0.096
Intercept	44.909	1	44.909	1148.560	0.000
Lipid levels	0.280	1	0.280	7.148	0.017
Lipid sources	0.200	1	0.200	5.111	0.038
Protein levels	0.022	1	0.022	0.568	0.462
Lipid levels*Lipid sources	0.053	1	0.053	1.361	0.261
Lipid levels*Protein levels	0.000	1	0.000	0.005	0.943
Lipid sources*Protein levels	0.036	1	0.036	0.922	0.351
Lipid levels*Lipid sources*Protein levels	0.000	1	0.000	0.005	0.943
Error	0.626	16	0.039		
Total	46.126	24			
Corrected Total	1.217	23			

a, R Squared = 0.486 (Adjusted R Squared = 0.261)

Appendix 2.17 Estimated marginal means of feed protein efficiency ratio

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		1.476	0.057	1.355	1.597
12%		1.260	0.057	1.139	1.381
Lipid sources					
Fish oil		1.277	0.057	1.156	1.398
Soybean oil		1.459	0.057	1.338	1.580
Protein levels					
35%		1.338	0.057	1.216	1.459
40%		1.398	0.057	1.277	1.519
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	1.432	0.081	1.261	1.603
	Soybean oil	1.520	0.081	1.349	1.691
12%	Fish oil	1.122	0.081	0.951	1.293
	Soybean oil	1.398	0.081	1.227	1.569
Lipid levels*Protein levels					
8%	35%	1.448	0.081	1.277	1.619
	40%	1.503	0.081	1.332	1.674
12%	35%	1.227	0.081	1.056	1.398
	40%	1.293	0.081	1.122	1.464
Lipid sources*Protein levels					
Fish oil	35%	1.285	0.081	1.114	1.456
	40%	1.268	0.081	1.097	1.439

Appendix 2.17 Estimated marginal means of feed protein efficiency ratio (continue)

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Soybean oil	35%		1.390	0.081	1.219	1.561
	40%		1.528	0.081	1.357	1.699
Lipid levels*Lipid sources*Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	1.440	0.114	1.198	1.682
		40%	1.423	0.114	1.181	1.665
	Soybean oil	35%	1.457	0.114	1.215	1.699
		40%	1.583	0.114	1.341	1.825
12%	Fish oil	35%	1.130	0.114	0.888	1.372
		40%	1.113	0.114	0.871	1.355
	Soybean oil	35%	1.323	0.114	1.081	1.565
		40%	1.473	0.114	1.231	1.715

Appendix 2.18 ANOVA of protein productive value of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	171.596[a]	7	24.514	2.609	0.053
Intercept	11073.370	1	11073.370	1178.414	0.000
Lipid levels	91.026	1	91.026	9.687	0.007
Lipid sources	55.146	1	55.146	5.869	0.028
Protein levels	6.448	1	6.448	0.686	0.420
Lipid levels*Lipid sources	11.043	1	11.043	1.175	0.294
Lipid levels*Protein levels	0.558	1	0.558	0.059	0.811
Lipid sources*Protein levels	7.150	1	7.150	0.761	0.396
Lipid levels*Lipid sources*Protein levels	0.224	1	0.224	0.024	0.879
Error	150.349	16	9.397		
Total	11395.315	24			
Corrected Total	321.946	23			

a, R Squared = 0.533 (Adjusted R Squared = 0.329)

Appendix 2.19 Estimated marginal means of protein productive value

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		23.428	0.885	21.552	25.303
12%		19.533	0.885	17.657	21.408
Lipid sources					
Fish oil		19.964	0.885	18.088	21.840
Soybean oil		22.996	0.885	21.120	24.872
Protein levels					
35%		20.962	0.885	19.086	22.838
40%		21.998	0.885	20.122	23.874
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	22.590	1.251	19.937	25.243
	Soybean oil	24.265	1.251	21.612	26.918
12%	Fish oil	17.338	1.251	14.685	19.991
	Soybean oil	21.727	1.251	19.074	24.380
Lipid levels*Protein levels					
8%	35%	23.062	1.251	20.409	25.715
	40%	23.793	1.251	21.140	26.446
12%	35%	18.862	1.251	16.209	21.515
	40%	20.203	1.251	17.550	22.856
Lipid sources*Protein levels					
Fish oil	35%	19.992	1.251	17.339	22.645
	40%	19.937	1.251	17.284	22.590

Appendix 2.19 Estimated marginal means of protein productive value

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Soybean oil	35%		21.932	1.251	19.279	24.585
	40%		24.060	1.251	21.407	26.713
Lipid levels*Lipid sources*Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	22.867	1.770	19.115	26.619
		40%	22.313	1.770	18.561	26.065
	Soybean oil	35%	23.257	1.770	19.505	27.009
		40%	25.273	1.770	21.521	29.025
12%	Fish oil	35%	17.117	1.770	13.365	20.869
		40%	17.560	1.770	13.808	21.312
	Soybean oil	35%	20.607	1.770	16.855	24.359
		40%	22.847	1.770	19.095	26.599

Appendix 2.20 ANOVA of *in-vitro* protein digestibility of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.733[a]	7	0.248	3.704	0.043
Intercept	35.820	1	35.820	535.877	0.000
Lipid levels	0.127	1	0.127	1.901	0.205
Lipid sources	1.165	1	1.165	17.433	0.003
Protein levels	0.018	1	0.018	0.265	0.621
Lipid levels*Lipid sources	0.398	1	0.398	5.957	0.041
Lipid levels*Protein levels	0.018	1	0.018	0.263	0.622
Lipid sources*Protein levels	0.002	1	0.002	0.026	0.876
Lipid levels*Lipid sources*Protein levels	0.006	1	0.006	0.084	0.779
Error	0.535	8	0.067		
Total	38.088	16			
Corrected Total	2.268	15			

a, R Squared = 0.764 (Adjusted R Squared = 0.558)

Appendix 2.21 Estimated marginal means of *in-vitro* protein digestibility

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		1.585	0.091	1.375	1.796
12%		1.407	0.091	1.196	1.618
Lipid sources					
Fish oil		1.226	0.091	1.016	1.437
Soybean oil		1.766	0.091	1.555	1.977
Protein levels					
35%		1.463	0.091	1.252	1.674
40%		1.530	0.091	1.319	1.740
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	1.473	0.129	1.175	1.771
	Soybean oil	1.698	0.129	1.399	1.996
12%	Fish oil	0.980	0.129	0.681	1.278
	Soybean oil	1.835	0.129	1.537	2.133
Lipid levels*Protein levels					
8%	35%	1.585	0.129	1.287	1.883
	40%	1.586	0.129	1.287	1.884
12%	35%	1.341	0.129	1.043	1.639
	40%	1.474	0.129	1.175	1.772
Lipid sources*Protein levels					
Fish oil	35%	1.183	0.129	0.885	1.481
	40%	1.270	0.129	0.972	1.568

Appendix 2.21 Estimated marginal means of *in-vitro* protein digestibility (continue)

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Soybean oil	35%		1.743	0.129	1.445	2.041
	40%		1.789	0.129	1.491	2.087
Lipid levels*Lipid sources*Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	1.444	0.183	1.022	1.866
		40%	1.503	0.183	1.081	1.924
	Soybean oil	35%	1.727	0.183	1.305	2.148
		40%	1.669	0.183	1.247	2.090
12%	Fish oil	35%	0.922	0.183	0.500	1.343
		40%	1.038	0.183	0.616	1.459
	Soybean oil	35%	1.760	0.183	1.338	2.182
		40%	1.910	0.183	1.488	2.331

Appendix 2.22 ANOVA of *in-vivo* protein digestibility of experiment 4

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	49.963[a]	7	7.138	11.107	0.000
Intercept	151748.016	1	151748.016	236133.15	0.000
Lipid levels	20.406	1	20.406	31.753	0.000
Lipid sources	2.714	1	2.714	4.223	0.057
Protein levels	7.315	1	7.315	11.383	0.004
Lipid levels*Lipid sources	0.076	1	0.076	0.118	0.736
Lipid levels*Protein levels	7.426	1	7.426	11.555	0.004
Lipid sources*Protein levels	8.178	1	8.178	12.726	0.003
Lipid levels*Lipid sources*Protein levels	3.848	1	3.848	5.988	0.026
Error	10.282	16	0.643		
Total	151808.261	24			
Corrected Total	60.245	23			

a, R Squared = 0.829 (Adjusted R Squard = 0.755)

Appendix 2.23 Estimated marginal means of *in-vivo* protein digestibility

				95% confidence Interval	
		Mean	Std. Error	Lower Bound	Upper Bound
Lipid levels					
8%		80.438	0.231	79.948	80.929
12%		70.594	0.231	78.104	79.085
Lipid sources					
Fish oil		79.180	0.231	78.689	79.671
Soybean oil		79.853	0.231	79.362	80.343
Protein levels					
35%		78.964	0.231	78.474	79.455
40%		80.068	0.231	7.578	80.559
Lipid levels*Lipid sources					
Lipid levels	Lipid sources				
8%	Fish oil	80.158	0.327	79.465	80.852
	Soybean oil	80.718	0.327	80.025	81.412
12%	Fish oil	78.202	0.327	77.508	78.895
	Soybean oil	78.987	0.327	78.293	79.680
Lipid levels*Protein levels					
Lipid levels	Protein levels				
8%	35%	79.330	0.327	78.636	80.024
	40%	81.547	0.327	80.853	82.240
12%	35%	78.598	0.327	77.905	79.292
	40%	78.590	0.327	77.896	79.284

Appendix 2.23 Estimated marginal means of *in-vivo* protein digestibility (continue)

					95% confidence Interval	
			Mean	Std. Error	Lower Bound	Upper Bound
Lipid sources*Protein levels						
Lipid sources	Protein levels					
Fish oil	35%		79.212	0.327	78.518	79.905
	40%		79.148	0.327	7.455	79.842
Soybean oil	35%		78.717	0.327	78.023	79.410
	40%		80.988	0.327	80.295	81.682
Lipid levels*Lipid sources*Protein levels						
Lipid levels	Lipid sources	Protein levels				
8%	Fish oil	35%	79.233	0.463	78.252	80.214
		40%	81.083	0.463	80.102	82.064
	Soybean oil	35%	79.427	0.463	78.446	80.408
		40%	82.010	0.463	81.029	82.991
12%	Fish oil	35%	79.190	0.463	78.209	80.171
		40%	77.213	0.463	76.232	78.194
	Soybean oil	35%	78.007	0.463	77.026	78.988
		40%	79.967	0.463	78.986	80.948

VITAE

Name Mrs. Duangrat Chookird

Student ID 4643007

Educational Attainment

Degree	Name of Institution	Year of Graduation
B.Sc.(Aquatic Sciences)	Prince of Songkla University	1991
M.Sc.(Food Technology)	Prince of Songkla University	1995
M.M.	Walailak University	2006

Scholarship Awards during Enrolment

- Prince of Songkla University
- The Center for Agricultural Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education

List of Publications and Proceedings

Publications

Chookird, D., Tantikitti, C., Pongdara, A. and Srichanun, M. 2010. The effect of hemoglobin powder substituted for fishmeal on growth performance, protein digestibility and trypsin gene expression in *Litopenaeus vannamei*. Songklanakarin J. Sci. Technol. 32:119-127.

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Tantikitti, C., Chookird, D. and Srichanun, M. 2008. Effects of fishmeal substitution with hemoglobin powder on growth performance and protein digestibility in *Litopenaeus vannamei*. The 6th Regional IMT-GT Uninet Conference 2008, 28-30 August 2008, The Gurney Resort Hotel & Residences, Penang, Malaysia.