การปรับปรุงคุณภาพพ่อแม่พันธุ์หอยหวาน *Babylonia areolata* ด้วยอาหารเสริมกรดไขมันไม่อิ่มตัวสูง

นายสราวุธ แสงสว่างโชติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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QUALITY IMPROVEMENT OF SPOTTED BABYLON (*Babylonia areolata*) BROODSTOCK WITH HIGHLY UNSATURATED FATTY ACID-SUPPLEMENTED FEED

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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Department of Marine Science

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Thesis Title	Quality improvement of spotted babylon (*Babylonia areolata*) broodstock with highly unsaturated fatty acid–supplemented feed
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สราวุธ แสงสว่างโชติ: การปรับปรุงคุณภาพพ่อแม่พันธุ์หอยหวาน *Babylonia areolata* ด้วยอาหารเสริมกรดไขมันไม่อิ่มตัวสูง. (QUALITY IMPROVEMENT OF SPOTTED BABYLON (*Babylonia areolata*) BROODSTOCK WITH HIGHLY UNSATURATED FATTY ACID–SUPPLEMENTED FEED) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สมเกียรติ ปิยะธีรธิติวรกุล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร. นิลนาจ ชัยธนาวิสุทธิ์, 101 หน้า.

การศึกษาผลของกรดไขมัน และกรดไขมันอะราคิโดนิกต่อความสามารถในการสืบพันธุ์ของพ่อแม่พันธุ์หอยหวานโดยแบ่งการทดลองเป็น 2 การทดลอง การทดลองแรกใช้เวลา 120 วัน ประเมินความสามารถการสืบพันธุ์ คุณภาพของไข่และตัวอ่อน และกรดไขมันของไข่หอยหวาน เมื่อให้อาหารธรรมชาติคือ ปลาข้างเหลืองและอาหารผสม 4 สูตรที่มีระดับไขมัน 5% และ15% จากน้ำมันปลาทูน่า (TO) และน้ำมันผสมของน้ำมันปลาทูน่ากับน้ำมันถั่วเหลือง (MO) (อัตราส่วน 3:2) ผลของอาหารควบคุม (เนื้อปลา) พบว่าระดับกรดไขมัน 20:5n-3, 22:6n-3 และ 20:4n-6ในฝักไข่มีค่าต่ำ เมื่อเปรียบเทียบกับอาหารสูตรอื่น โดยสูตรอาหาร 5%TO ให้ผลของกรดไขมันในฝักไข่สูงที่สุด และความสามารถการสืบพันธุ์สูงที่สุดพบในหอยเพศเมียที่ได้รับอาหารสูตร 5%TO แต่ผลของคุณภาพของไข่และตัวอ่อน ระยะเวลาการทดสอบความทนทานต่อการอดอาหารไม่ต่างกัน และค่ากรดไขมันสำคัญในฝักไข่จากแม่พันธุ์ที่กินอาหารสูตร 5%TO มีความแตกต่างอย่างมีนัยสำคัญทางสถิติสูงกว่าอาหารอื่น อาหารผสมที่เติมน้ำมันปลาทูน่าให้ผลดีต่อการวางไข่และทำให้ฝักไข่มีระดับของกรดไขมันสำคัญสูงขึ้นเมื่อเปรียบเทียบกับการใช้เนื้อปลา สำหรับการทดลองที่สองได้ศึกษาผลของระดับกรดอะราคิโดนิกต่อความสามารถในการวางไข่ คุณภาพของไข่และตัวอ่อน และองค์ประกอบของกรดไขมันในไข่หอยหวาน โดยใช้อาหาร 5 สูตร ที่มีส่วนผสมของอาหารเหมือนกัน แต่มีการเสริมปริมาณของกรดอะราคิโดนิก (ARA) ต่างกัน 5 ระดับ (0%, 0.4%, 0.8%, 1.2% และ 1.6%) ผลการทดลองพบว่าอาหารเสริม ARA มีผลต่อการวางไข่ (จำนวนการวางไข่รวมและความถี่การออกไข่ต่อเดือน) โดยจำนวนครั้งของการวางไข่และความถี่ในการออกไข่ต่อเดือนมีค่าเพิ่มขึ้นตามการเพิ่มขึ้นของระดับ ARA โดยหอยเพศเมียที่กินอาหารสูตร 5 ให้ผลการวางไข่สูงสุด (25.5 ครั้ง), สูตร 4 (23.0 ครั้ง), สูตร 3 (22.0 ครั้ง), สูตร 1 (17.5 ครั้ง) และ สูตร 2 (15.5 ครั้ง) สำหรับผลคุณภาพของไข่ (ตัวอ่อนในฝักไข่, ขนาดของฝักไข่, ระยะเวลาในการฟัก และอัตราการฟัก) ไม่มีความสัมพันธ์กับการเสริม ARA นอกจากนี้คุณภาพของลูกหอยวัยอ่อน (ความทนทานต่อความเค็มต่ำและความทนทานต่อการอดอาหารของลูกหอยวัยอ่อน) ไม่มีค่าเพิ่มขึ้นตามการเพิ่มขึ้นของระดับ ARA การศึกษาในครั้งนี้สามารถสรุปได้ว่าการเสริมระดับ ARA ในปริมาณสูงมีผลต่อคคุณภาพการวางไข่ของหอยหวาน แต่ไม่มีผลต่อคุณภาพของไข่และตัวอ่อนและองค์ประกอบของกรดไขมันในฝักไข่

สาขาวิชา วิทยาศาสตร์ทางทะเล ลายมือชื่อนิสิต.....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4873857423 : MAJOR MARINE SCIENCE
KEYWORDS **:** BROODSTOCK/ *Babylonia areolata*/ REPRODUCTIVE PERFORMANCE/ CONDITIONING DIETS/ FATTY ACID, ARACHIDONIC ACID/ EGG AND LARVAE QUALITY

SARAWUT SANGSAWANGCHOTE : QUALITY IMPROVEMENT OF SPOTTED BABYLON (*Babylonia areolata*) BROODSTOCK WITH HIGHLY UNSATURATED FATTY ACID–SUPPLEMENTED FEED. THESIS ADVISOR : ASSOC. PROF. SOMKIAT PIYATIRATITIVORAKUL, Ph.D., THESIS CO-ADVISOR : NILNAJ CHAITANAWISUTI, Ph.D., 101 pp.

This study was designed to study effects of fatty acids and arachidonic acid on reproductive performance of hatchery-reared broodstock spotted babylon, *Babylonia areolata*. The study was divided into two experiments. The first experiment, a 120-day feeding trial was conducted to evaluate reproductive performance, egg and larval quality and egg fatty acid composition in spotted babylon broodstock fed natural food (fresh meat of carangid fish, *Selaroides leptolepis*,) and four experimental formulated diets containing 5% or 15% of dietary lipid from tuna oil (TO) and a mixture of tuna oil and soybean oil (MO) (ratio 3:2) labeled as 5%TO, 15%TO, 5%MO and 15%MO respectively. Using trash carang resulted the lowest levels of in 20:5n-3, 22:6n-3 and 20:4n-6 fatty acids compared to those of all experimental diets. The highest content of total fatty acids was found in the 5%TO diet. The best reproductive performance were found only for females fed the 5%TO diets, but egg and larval quality showed no variability among females fed. No significant differences were observed in survival duration in the starvation tolerance test for females fed trash fish or any of the experimental diets. However, the fatty acid profile of egg capsules was significantly affected by the dietary treatments. The levels of major fatty acids (20:5n-3, 22:6n-3 and 20:4n-6) in egg capsules produced from females fed diets containing 5% tuna oil (5%TO) was significantly higher than those females fed trash fish or other experimental diets. We therefore conclude that formulated diets with fish oil resulted in successful reproduction and high essential fatty acids in egg capsules comparable to the use of trash fish. The second experiment studied on the effects of arachidonic acid in broodstock diet on spawning performance, egg and larval quality and fatty acid composition of eggs from broodstock spotted babylon (*B. areolata*). A formulated diet containing similar compositions was supplemented with five levels of arachidonic acid (20:4n-6, ARA); 0% (1), 0.4% (2), 0.8% (3), 1.2% (4) and 1.6% (5) ARA, respectively. Results showed that spawning quality (total number of spawning and monthly spawning frequency) were affected by ARA supplementation. The total number of spawning and monthly spawning frequency throughout the experiment increased with an increased supplemented of ARA levels. Females fed on diet 5 had the highest total number of spawn (25.5), followed by the diet 4 (23.0), diet 3 (22.0), basic diet (17.5) and diet 2 (15.5). While the egg quality (number of fertilized eggs in capsule, length and width of egg capsules, egg incubation time and hatching rate) were not affected by ARA supplementation. For larval quality, ARA did not enhance tolerance to low salinity stress and starvation test together with increasing the levels of dietary ARA in *B. areolata* broodstock. These results indicated that high level of dietary arachidonic had a positive effect on spawning performance of *B. areolata* broodstock and high level of arachidonic improved spawning performance but not for egg and larval quality and fatty acid composition of egg capsules.

Department : Marine Science Student's Signature

Field of Study : Marine Science Advisor's Signature

Academic Year : 2009 Co-Advisor's Signature

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CHAPTER I

INTRODUCTION

General background Information

The spotted babylon, *Babylonia areolata* Link, 1807, is now the most important marine gastropod for human consumption in Thailand, where the larger-sized (>450 mm) are used for the fried and steamed spotted babylon in sea food restaurants. Spotted babylon is abundant and widely inhabits littoral regions in the Gulf of Thailand, especially in the muddy sand areas of which not exceeding 10-20 m depth. However, natural stocks decrease widely from year to year due to continuous exploitation in various traditional fishing areas, confronting to the chronic supply shortage particularly in the larger size classes. Decreased production results in increasing in price and demand. It has come under significant study in recent years to protect spotted babylon as an important renewable marine resource and to increase market supply. Spotted babylon have been the subject of recent studies, particularly on their fishery and aquaculture because of their economic importance and decreasing natural stocks. One possible solution to over-exploitation is juvenile production in hatcheries for reseeding in natural areas. For spotted babylon aquaculture, the remarkable success of spotted babylon cultivation has instigated a widespread interest in developing the appropriate aquaculture systems as a mean of stock enhancement and increasing market supply. From an aquaculture point of view, spotted babylon has many desirable biological attributes for profitable aquaculture production, thus spotted babylon is now promising as a new candidate for aquaculture in Thailand.

Considerable interest has been recently developed regarding the commercial culture of spotted babylon in Thailand due to a growing demand and an expanding of domestic and export market. Early attempts to produce spotted babylon seedling met with failure more frequently than success. Although hatchery-based cultivation for large-scale production of spotted babylon is considered to be technically feasible, the successful techniques in the laboratory were not directly transferable to the commercial operations, where large numbers of spotted babylon seedling needed to be produced in high volume, intensive, factory-like systems. As spotted babylon culture is a nascent industry compared with many marine bivalve and gastropod cultures, further applied research is then necessary to develop an economically viable culture operation for decreasing the maintenance time and production costs of spotted babylon during growing-out phase. However, few studies exist concerning the biology and culture of spotted babylon but these basic information is needed in order to develop optimal rearing protocol for commercial spotted babylon culture in Thailand.

Justification of this study

The spotted babylon, *B. areolata*, have been identified as important candidates for aquaculture in Thailand. In recent year, some information has been published on the biology and ecology of spotted babylon and considerable progress has been made in their aquaculture potentials. Techniques have now been developed to culture for all stages of the life cycle of spotted babylon. The most critical period in the production of spotted babylon is the larval stage. After hatching, the larvae need to be fed the live feed. Consequently, at the end of the larval life stage, when the larvae metamorphose into juveniles, the live food is replaced by dead fish or shellfish. It is generally thought that most technical problems must be solved in the larval rearing phase of this

species. The survival rates from the hatching of the eggs until the weaning of the larvae are not always stable, and mass mortality of larvae is common. Although, considerable progress has been made during the past few years, the production of juveniles is still one of the main bottlenecks for this species. The problems seem to be the same for many species of both fish and shellfish, with poor reproducibility in terms of survival, growth and quality as the main symptoms. This suggests that there is a lack of investigation on various limiting factors upon growth and survival of larvae and juveniles of this species. Among these factors, the effects of food items, stocking density and egg quality seem to be ruled out as the principal cause. However, the hatchery-based system for commercial operations is needed to be developed for large numbers of spotted babylon seedling production in high volume, intensive, factory-like systems.

Present problems

A major constraint to the development of the spotted Babylon, *B. areolata*, aquaculture in Thailand is the insufficient supply of seed and high cost production. Successful conditioning of broodstock *B. areolata* is still a crucial step for selective breeding programs to produce a large quantity of eggs and larvae of good quality for the growing industrial importance of this species in Thailand because large variability in spawning events, hatchability, and larval and juvenile survival rates of the spotted babylon has been observed during the same season between batches and hatcheries. This variability remained high despite each batch of larvae being reared in a standardized manner which included the control of larval density, water management and the use of selected microalgal species. Production of good quality larvae is very inconsistent (Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu, 1997). One of the main reasons for unpredictable larvae culture outputs is the variable quality of eggs and

larvae. Several factors affect egg and larvae quality in fish and shellfish species. These are either endogenous (genotype, age, and size of broodstock, egg size) or exogenous (egg management, broodstock feeding, bacterial colonisation of egg surface (Ballestrazzi et al., 2003). In teleosts, nutrients such as protein, fatty acids, vitamin E, ascorbic acids and carotenoids have been implicated in various reproductive-related processes such as gonadal maturation, gamete quality and spawning performances. Interaction between nutrients and reproductive processes, however, remains poorly understood. Several studies have highlighted the importance of both quantity and quality of dietary lipid on reproductive performances of broodstock (Ling et al., 2006). Teruel, Millamena and Fermin (2001) reported that a higher amount of essential nutrients such as protein, lipid and the highly unsaturated fatty acid, e.g. 20:4n-6, 20:5n-3, 22:6n-3 in the artificial diet influenced the increased reproductive performance for abalone, *Haliotis asinina*. Utting and Millican (1997) reported that the number of eggs produced and polyunsaturated fatty acid (PUFA) composition of the eggs of marine bivalves (scallops, oysters and clams) are influenced by the quantity and quality of lipid in microalgae diet supplements. Under optimal hatchery rearing conditions, differences in initial egg lipid reserves may not necessary affect subsequent larval growth and survival. In addition, the importance of lipid and PUFA reserves, in particular, eicosapentaenoic acid, on 20:5n-3, during the development of embryos and larvae can, however, be clearly demonstrated under more stressful rearing conditions. It remains unclear which constituents are responsible for triggering maturation, egg laying of broodstock, therefore, more detailed research on reproductive performance is needed. There are no published studies on the influence of nutrition on the reproductive performance of spotted babylon broodstock, despite their importance in commercial aquaculture. Thus, there is a need to develop a

reliable technique for spotted babylon broodstock development through dietary manipulation.

Concept of this study

One of the priorities for spotted babylon farming in Thailand is the development of a fully formulated broodstock diet, which would provide good spawning, and egg and larval quality but be free of the trash fish components traditionally used by the farmer.

- Nutritional composition and quality of a broodstock diet is an important contributor to egg and larval quality, since during ovarian development, dietary and maternal reserves are mobilised and transported into the oocytes, where they provided the energy and nutritional requirements for growth and development of the embryo and the yolk sac larvae up till starting of exogenous feeding.

- Broodstock nutrition affects reproduction and egg quality and some feed components are stress to greatly influence spawning quality in several fish species. Several studies have highlighted the importance of both quantity and quality of dietary lipid on reproductive performances of broodstock.

- Essential fatty acids are one of the nutritional factors, which greatly affect spawning, egg and larval quality. More recently, attention has partly shifted to include arachidonic acid (ARA: 20:4n-6), mainly because of its role in eicosanoid production and the involvement of eicosanoids in a range of physiological functions, including reproduction and egg development.

Targets of this study

This research targeted to solve the main problems concerning the broodstock maintenance and conditioning in commercial hatcheries as following:

1) To develop the appropriate formulated broodstock diets, which provide good spawning, and egg and larval quality as well as replacement the use of trash fish traditionally used by the commercial farms.

2) To extend the use of new broodstock last longer in commercial hatcheries because of their high expenses and unsufficience in supply.

3) To improve the linger used broodstock in commercial hatcheries for better spawning quality and high quality of egg and larvae.

Objectives of this study

This dissertation targeted to provide information as a guideline for development of appropriate practical diets for broodstock of *Babylonia areolata* in commercial scales. Consequently, this study was focus on the improvement of broodstock quality of *B. areolata* providing higher quality of eggs and larvae for hatchery operations. Then, the present study had the special emphasis targets on two parts of broodstocking conditioning diets as following:

Part 1: Reproductive performance, egg and larval quality and egg fatty acid composition of hatchery-reared spotted babylon (*B. areolata*) broodstock fed natural and formulated diets under hatchery conditions

Part 2: Effects of arachinodic acid levels in broodstock diets on spawning performance, egg and larval quality and fatty acid composition of eggs and broodstock spotted babylon (*B. areolata*)

CHAPTER II

LITERATURE REVIEW

Biological aspects of the spotted babylon

The spotted babylon, *Babylonia areolata* Link, 1807, belongs to the Phylum Mollusca, Class Neogastropoda, Order Neogastropoda and Family Buccinidae. The Order Neogastropoda is very large, with about 5,000 species and almost all members of this order are marine benthic and carnivores. The family Buccinidae is one of the most diverse and dominant groups of predatory gastropods that distribute around area of the seabed. *B. areolata* shell is relatively thin but solid, elongate-ovate in shape, with a high, conical spire and large, inflated body, spire whorls convex, distinctly shouldered below the sutures. Outer surface is smooth and polished, white, with three spiral rows of large, quadrate brown spots on body whorl, and one row of such spots on spire whorls, operculum large and thick (Figure 2-1). The genus *Babylonia* is endemic to the Indo West Pacific area (Habe, 1965). This species distributes from Ceylon and the Nicobar Islands through the Gulf of Thailand, along the Vietnamese and Chinese coasts to Taiwan. In Thai seacoast, *B. areolata* is widely distributed, abundant and inhabits in littoral regions in depth from 5 to 15 m in the Gulf of Thailand such as the coastal waters of Rayong, Trad, Petchaburi, Prachuab Khiri Khan, and Chumphon, provinces. Spotted babylon prefers the environment with salinity ranging from 28-35 ppt and water temperature of 25-30^{o} C. They were found in variety of habitats such as fine sand, coarse sand, sandy mud, and muddy sand. Spotted babylon spends most of their life buried in the substrate with full or half bury in substrate of its shell height then their distribution is limited by substrate in natural habitats.

Figure 2-1. The spotted babylon (*B. areolata*, Link 1807) (Nilnaj Chaitanawisuti, 2010 : online)

The reproductive organs and sex differentiations of the male and female spotted babylon *B. areolata* were demonstrated in Figure 2-2 and Figure 2-3. Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu (1997) reported that the spotted babylon lay egg naturally under hatchery conditions all year round with maximum in summer period during February – August (Figure 2-4). Broodstock with average shell length of 5.0 – 7.0 cm spawned naturally during early morning. Most egg capsules were individually attached to the sand substratum by a long, narrow stalk. Egg capsules were moderately transparent and vasiform in shape, they were broad at the apex and narrow toward the base. Each capsule possesses a short stalk (peduncle) that is cemented to the substrate. The fertilized eggs are visible and suspended in albuminous fluid inside the capsule. Egg capsules averaged 21.43$\pm$1.3 mm in length and 9.57$\pm$1.0 mm in width. An average female spotted babylon (5.7 cm long) spawned 46 egg capsules (rang = 22 - 57). The average egg number per capsule was 851 eggs (range =

493 – 1,133), and the average egg diameter was 425.70 μm. Spotted babylon fecundity averaged 39,146 eggs per individual.

Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu (1997) demonstrated the life cycle of the spotted babylon (*B. areolata*) as showed in Figure 2-5. The fertilized eggs are visible and suspended in albuminous fluid inside the capsule. The trochophore larvae were developed from single cell to early veliger stage inside egg capsules during the first 5 days. The veliger hatched through the apical opening into the water column within 5 days after spawning. Veliger larvae were hatched at 28 to 30 ppt salinity and 28 to 31^{o}C water temperature. The average hatching rate was 95.0%. The newly-hatched veliger larvae had a transparent, thin shell and two large, lobed velum. The average shell length of veligers was 720.4$\pm$1.52 μm. After hatching, veligers were positively phototactic and planktotrophic. At day 9, the larval lobes became enlarged, with shell visible, and the larvae were about 870 μm long. The larvae settled to the bottom at about 1,540 μm. By day 16, the presence of a foot and swimming near the bottom were the first indications that the larvae were competent to settle. Metamorphosis juvenile was completed by day 14-16, and the juveniles averaged 1,520$\pm$1.64 μm long and 1,160$\pm$1.36 μm wide. Larvae metamorphosed and settled in the absence of substratum. The juveniles changed their behavior from herbivorous to be carnivorous, and they started feeding the fish meat at first day after settlement. During the period of settlement, heavy mortality occurred because the newly-settled juveniles changed the behavior from swimming to be crawling by mean of muscular foot, and they continually crawled out of the water and died as a result of dessication. The newly settled juveniles have to be cultured in nursery tanks until they reached the shell length of 0.5 cm, and they were collected for growing-out to marketable sizes.

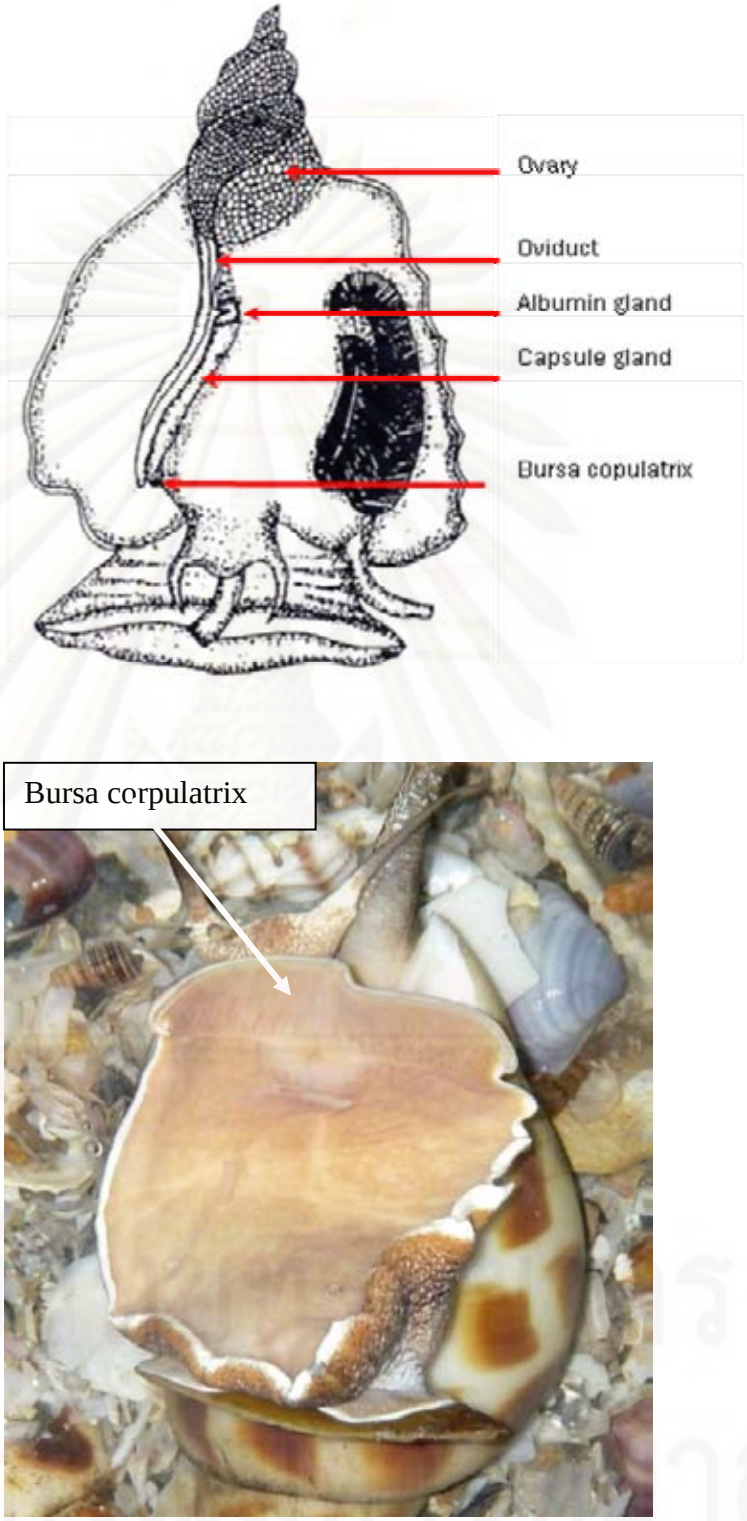


Figure 2-2. Reproductive organs (upper) and sex differentiation (lower) of female spotted babylon *B. areolata* (Nilnaj Chaitanawisuti, 2010 : online)

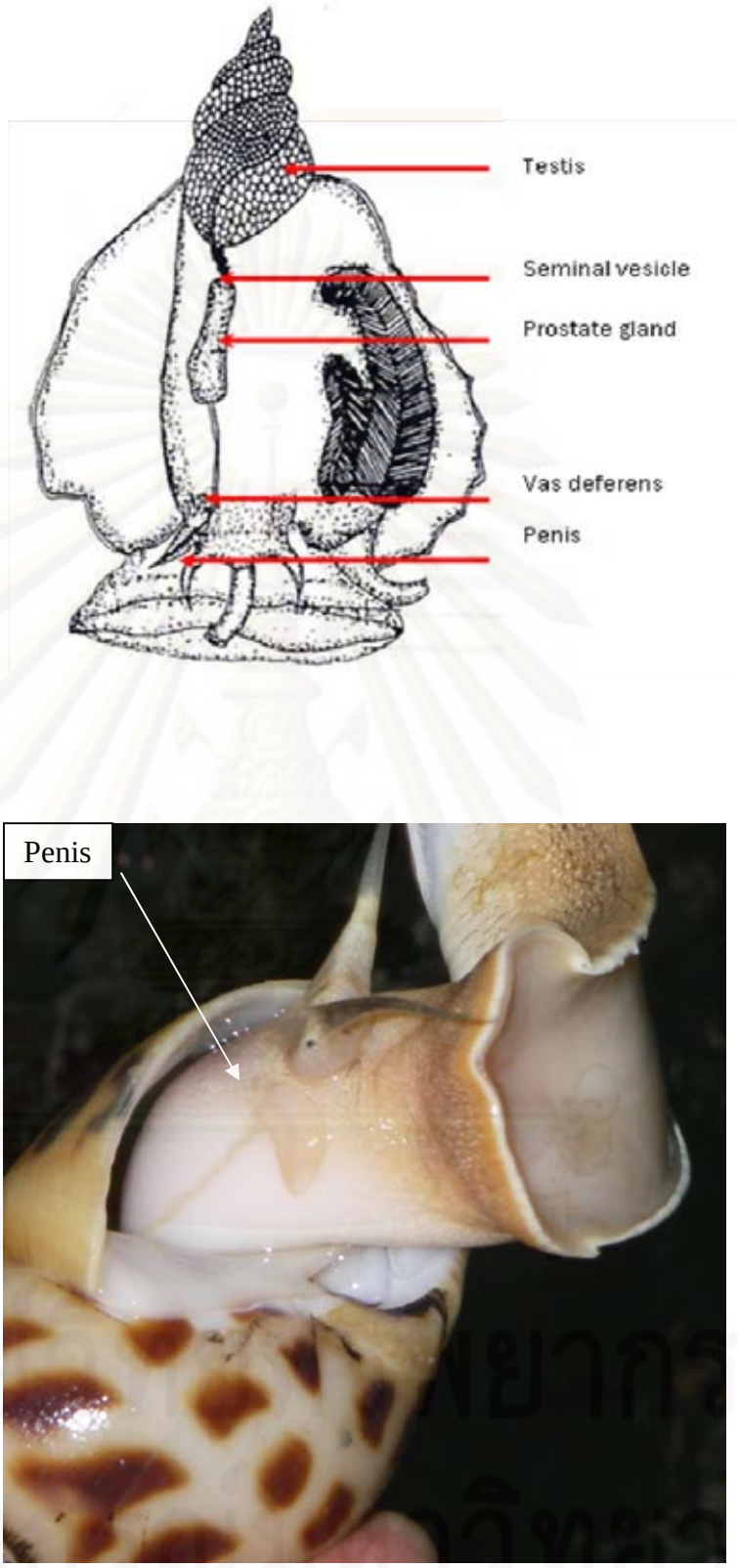


Figure 2-3. Reproductive organs (upper) and sex differentiation (lower) of male spotted babylon *B. areolata* (Nilnaj Chaitanawisuti, 2010 : online)

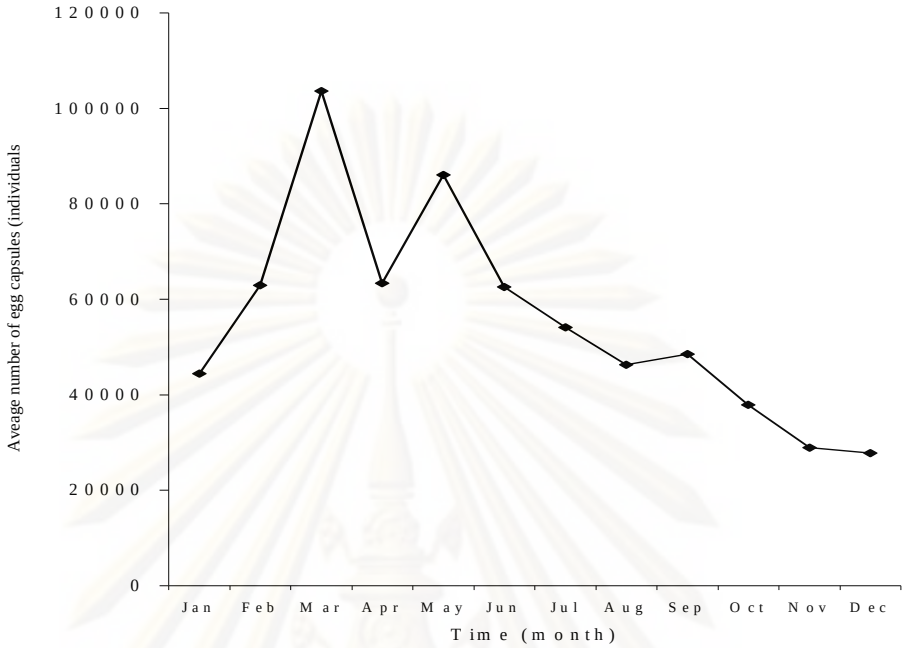


Figure 2-4. Egg laying of broodstock spotted babylon under hatchery conditions

(Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu, 1997)

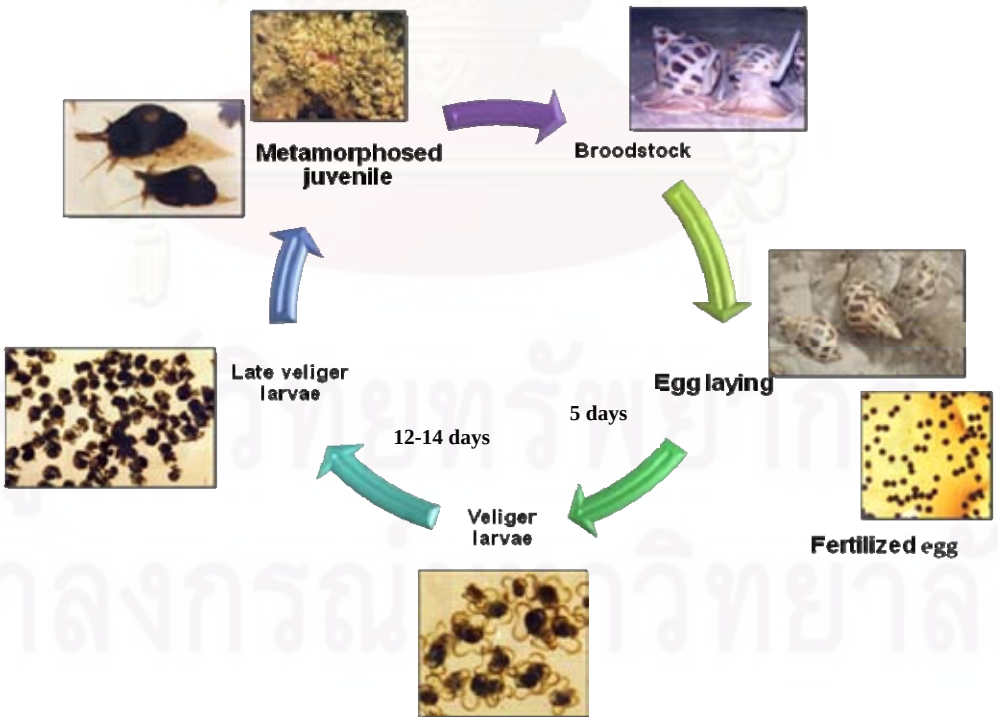


Figure 2-5. Life cycle of the *B. areolata* (Nilnaj Chaitanawisuti, 2010 : online)

Fatty acids and their roles in maturation and fecundity

Fatty acid is a carboxylic acid often with a long unbranched aliphatic tail (chain), which is either saturated or unsaturated. Carboxylic acids as short as butyric acid (4 carbon atoms) are considered to be fatty acids, whereas fatty acids derived from natural fats and oils may be assumed to have at least eight carbon atoms, caprylic acid (octanoic acid), for example. The most abundant natural fatty acids have an even number of carbon atoms because their biosynthesis involves acetyl-CoA, a coenzyme carrying a two-carbon-atom group (see fatty acid synthesis). Fatty acids are produced by the hydrolysis of the ester linkages in a fat or biological oil (both of which are triglycerides), with the removal of glycerol. Fatty acids are aliphatic monocarboxylic acids derived from, or contained in esterified form in, an animal or vegetable fat, oil, or wax. Fatty acids of animal origin are comparatively simple in structure and can be subdivided into well-defined families (Christie,1982). Natural fatty acids commonly have a chain of 4 to 28 carbons (usually unbranched and even numbered), which may be saturated or unsaturated. By extension, the term is sometimes used to embrace all acyclic aliphatic carboxylic acids. It is proposed that the blends of fatty acids exuded by mammalian skin, together with lactic acid and pyruvic acid, are distinctive and enable animals with a keen sense of smell to differentiate individuals. Saturated fatty acids are a long-chain carboxylic acid that usually has between 12 and 24 carbon atoms that has no double bonds. Thus, saturated fatty acids are saturated with hydrogen (since double bonds reduce the number of hydrogens on each carbon). Example; lauric acid (12:C), Myristic acid (14:C), Palmitic acid (16:C), Stearic acid (18:C), Arachidic acid (20:C). Unsaturated fatty acids (Fig 2-6) are of similar form, except that one or more alkenyl functional groups exist along the chain, with each alkene substituting a single-bonded " $-CH_2-$

CH_2-" part of the chain with a double-bonded "-CH=CH-" portion (that is, a carbon double-bonded to another carbon). The two next carbon atoms in the chain that are bound to either side of the double bond can occur in a cis- or trans- configuration.

Fatty acids are mobilized from the neutral lipid reserves of fish adipose tissue during gonadogenesis and transferred via the serum to the liver where they are assembled into the egg specific lipoprotein, vitellogenin. Up to 60% of the free fatty acids mobilized, preferentially saturated and monounsaturated fatty acids, can be catabolized to provide metabolic energy for egg liproprotein biosynthesis. The remainder, preferentially n-3 polyunsaturated fatty acids (PUFA) and especially 22:6n-3, are incorporated into the phospholipid-rich vitellogenin which is transferred via the serum to the developing egg. The major egg phospholipid is in variably phosphatidycholine, and eggs with short and long incubation times have low and high levels of triacylglycerols respectively.

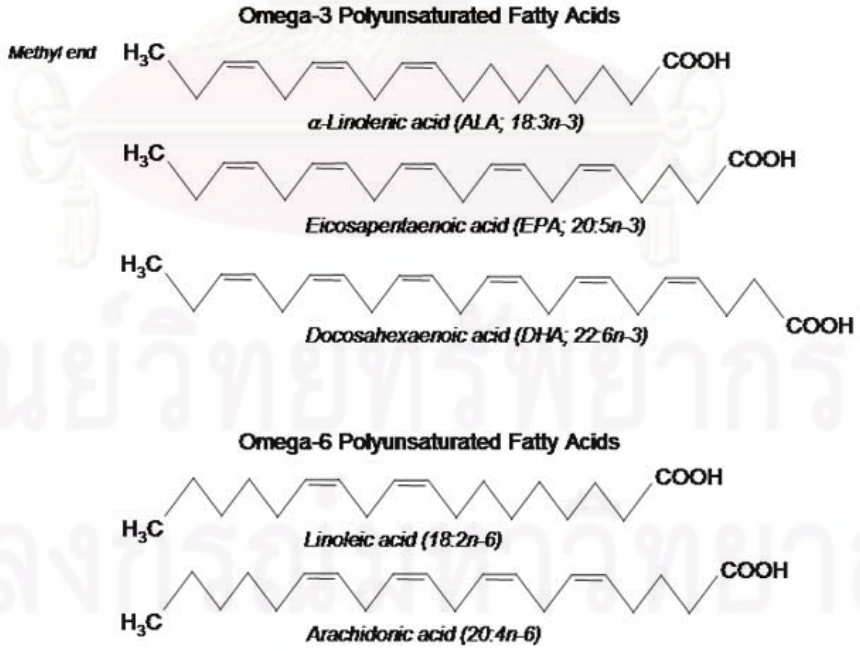


Figure 2-6. Structure of unsaturated fatty acids in omega 3 and omega 6 groups (Christie, 1982)

Phospholipid and triacylglycerols in eggs have levels of n-3 PUFA of circa 50% and 30% respectively, composed principally of 22:6n-3 and 20:5n-3 in a ratio of circa 2:1. The fatty acids of both phospholipid and triacylglycerols, including their n-3 PUFA, are catabolized to provide metabolic energy for the developing egg and early larva, but the chief role of n-3 PUFA is in the formation of cellular membranes. Because of the unusual richness of 22:6n-3 in neural cell membranes this fatty acid has a critical role in the formation of the brain and eyes, which constitute a large fraction of the embryonic and larval body mass. The small quantities of 20:4n-6 in fish eggs are located almost exclusively in phosphatidylinosital and specific role for this fatty acid in eicosanoid formation is indicated. From considerations of juvenile fish and analyses of fish eggs, an optimal level of n-3/n-6 PUFA in broodstock diets and in fish eggs of 5:1-10:1 indicated. The importance of using high quality marine fish oils in broodstock nutrition for successful normal embryonic and early larval development is stressed (Bromage and Roberts, 1995).

In aquaculture nutrition, docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6), collectively known as highly unsaturated fatty acids (HUFA) are widely studied in order to determine the optimal dietary requirement levels of broodstock, juvenile and larva of cultured species. DHA, EPA and ARA are broadly responsible for a generalized role in maintenance of cell membrane integrity. More specifically, these HUFA also serve as precursors for eicosanoids, an important group of paracrine hormones responsible for a whole range of physiological activities including development, immunity and reproduction. There is an urgent need to seek alternatives for utilization of marine-based oils, which are currently facing issues such as diminishing supply and escalating cost. Although a wide array of plant-based oils

have been suggested as suitable candidates, these oils usually possess very low or insufficient n-3 HUFA. Therefore, knowledge on the dietary HUFA requirements of cultured species will be crucial to facilitate attempts to effectively incorporate correct levels of plant oils as dietary lipid source. In comparison with marine fish species, which generally require substantial levels of dietary HUFA, freshwater species possess the ability to synthesize EPA and DHA from linolenic acid (LNA, 18:3n-3) and ARA from the linoleic acid (LA, 18:2n-6). This is due to the presence of desaturase and elongase enzymes responsible for two important steps in the HUFA biosynthesis pathway, desaturation and elongation (Ling, 2006). In teleosts, numerous nutrition related factors such as feed ration, nutrient levels and compositions have been shown to influence various reproductive parameters such as gonadal development, egg quantity and quality, spawning success, hatchability and larval quality. Provision of lipids to oocytes, followed by storage and accumulation in yolk and subsequent utilization by developing embryos is essential processes in reproduction and development. Studies in the past two decades involving variety of farmed fish species have identified lipid, and in particular HUFA, as key nutrients affecting broodstock reproductive performances. HUFA plays an integral role in regulating levels of eicosanoids, which in turn control selected stages of reproduction such as steroidogenesis and ovulation.

Marine fish are generally presumed to have a low capability or inability to bioconvert 18C fatty acids, i.e., linoleic (18:2n-6) and linolenic (18:3n-3) acids, into 20-22C HUFA. Therefore, HUFA are considered as essential fatty acid (EFA) required in their diets for normal growth and survival. The importance of HUFA, especially of n-3 HUFA (EPA and DHA), in broodstock nutrition has attracted attention during the last two decades. It has been well documented that dietary EFA,

especially n-3 HUFA, is one nutritional factor that greatly affects spawning performance. In general, low n-3 HUFA levels in broodstock diets decrease egg and larval quality. However, some authors have identified negative effects of excess n-3 HUFA on egg quality. The fatty acid composition of eggs is directly affected by the n-3 HUFA content of the broodstock diet. Some fish species readily incorporate dietary unsaturated fatty acids into eggs, even during the course of the spawning season. HUFA with 20 or more carbon atoms affect, directly or through their metabolites, fish maturation and steroidogenesis. In some species, HUFA in broodstock diets increases fecundity, fertilization and egg quality. As in higher vertebrates, vitamin E deficiency affects reproductive performance, causing immature gonads and lower hatching rate and survival of offspring (Izquierdo et al., 2001).

Several studies were conducted on effects of n-3 and n-6 fatty acids on broodstock quality, spawning quality and egg and larval quality in marine fish (Li et al. 2005; Furuita et al. 2007; Salze, et. al. 2005), shellfish (Cahu et al. 1994; Lytle et al. 1990) and mollusks (Palacios et al. 2005; Utting and Millican 1997; Utting and Millican 1998; Hurtado et al. 2009). Palacios et al. (2005) reported that accumulation of lipids in female gonads during gametogenesis is well documented for several species of pectinids. There are two postulated mechanisms by which pectinids accumulate lipids in the female gonads during maturation: (1) transfer of lipids from the digestive gland to the gonads, and (2) lipogenesis from carbohydrates stored in the muscle, which results in a decrease of muscle carbohydrates during gametogenesis. Lipogenesis or synthesis of saturated fatty acids can occur directly in the gonads or in other tissues. However, mollusks have a limited capacity for elongation and desaturation of long-chain polyunsaturated fatty acids (PUFA). Thus, PUFA for gonad development are probably obtained directly from diet or indirectly after

previous accumulation in the digestive gland or other tissues. The role of diet in comparison to previously stored components has been addressed for general biochemical composition, but there are few studies that analyze tissue-specific fatty acid variations in relation to gametogenesis in pectinids, and none for giant lion's-paw scallop. Utting and Millican (1997) reported that the number of eggs produced and PUFA composition of the eggs of marine bivalves (scallops, oysters and clams) are influenced by the quantity and quality of lipid in microalgae diet supplements. Under optimal hatchery rearing conditions, differences in initial egg lipid reserves may not necessary affect subsequent larval growth and survival. In addition, the importance of lipid and PUFA reserves, in particular, eicosapentaenoic acid, on 20:5n-3, during the development of embryos and larvae can, however, be clearly demonstrated under more stressful rearing conditions. In addition, Utting and Millican (1998) stated that when scallops are conditioned to spawn in the hatchery, the physical environment and the nutrition of broodstock animals are manipulated to promote gonad development and gametogenesis. Successful broodstock conditioning depends on providing conditions in the hatchery that approximate those that occur in the wild during the natural cycle of reproduction, namely manipulating seawater temperature and supplying adequate quantities of appropriate phytoplankton species. This ensures that broodstock produce the maximum number of high quality eggs which will develop into viable larvae with good rates of growth and survival.

More attention has recently shifted to arachidonic acid (ARA: 20:4n-6), because of its role in is a precursor for synthesis of eicosanoids, mainly prostaglandins of the series II (PG_2). Either way, an increase in PG_2 has been associated with gonad development and oocyte releasing during spawning in mollusk (Martinez *et al.* 1996, 2001). Significant improvements in egg and larval quality have been seen in Atlantic

halibut broodstock fed diets containing supplemental ARA. Fish fed diets containing 2% of ARA showed significant improvements in fertilisation and hatching rates compared to those containing 0.5% or 1.0% ARA. Arachidonic acid is freed from a phospholipid molecule by the enzyme phospholipase A_2 (PLA_2), which cleaves off the fatty acid, but can also be generated from DAG by Diacylglycerol lipase. Arachidonic acid generated for signaling purposes appears to be derived by the action of a phosphatidylcholine-specific cytosolic phospholipase A_2 ($cPLA_2$, 85 kDa), whereas inflammatory arachidonic acid is generated by the action of a low-molecular-weight secretory PLA_2 ($sPLA_2$, 14-18 kDa). Arachidonic acid is a precursor in the production of eicosanoids (Figure 2-7).

- The enzymes cyclooxygenase and peroxidase lead to Prostaglandin H_2 , which in turn is used to produce the prostaglandins, prostacyclin, and thromboxanes.

- The enzyme 5-lipoxygenase leads to 5-HPETE, which in turn is used to produce the leukotrienes.

- Arachidonic acid is also used in the biosynthesis of anandamide.

- Some arachidonic acid is converted into hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs) by epoxygenase.

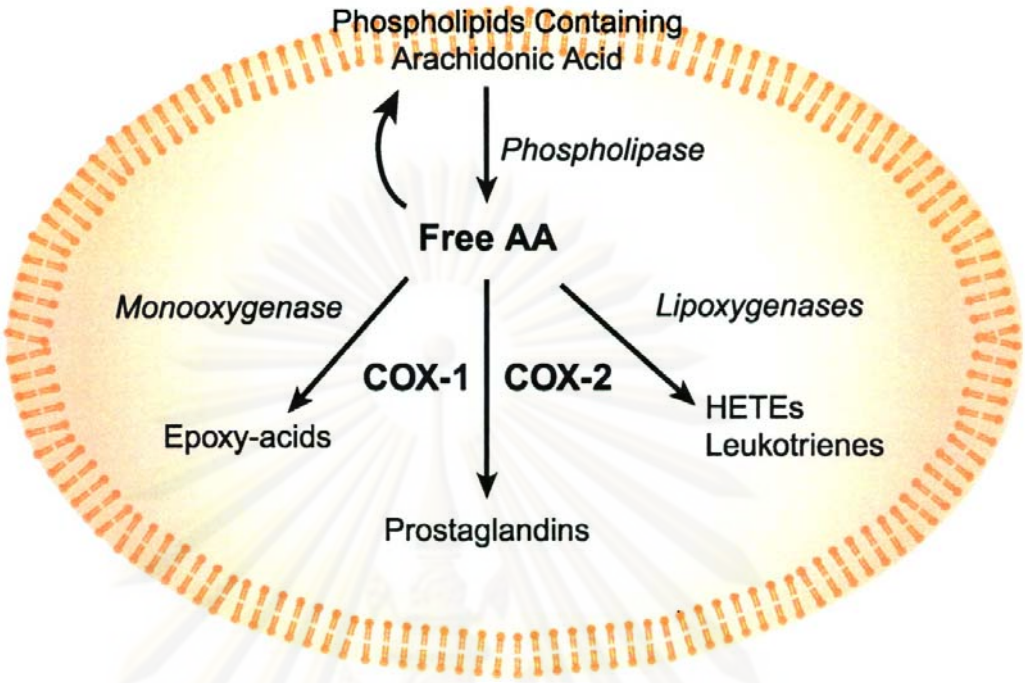
The effect of ARA on reproduction; egg, sperm and larval quality of marine fish. The importance of ARA in reproduction was first identified in European sea bass broodstock fed diets containing either northern hemisphere fish oil or a local trash fish, bogue (*Boops boops*). The trash fish diet contained around eightfold more ARA than the fish oil diet and the EPA/ARA ratios were 1.5 and 15 for the trash fish and fish oil diets, respectively. Dietary ARA was found to be highly concentrated in the eggs and sperm of sea bass broodstock fed trash fish and in wild-caught broodstock. Subsequently, pelleted dry feeds were prepared with increased levels (twofold) of

ARA which were successful in improving egg and larval quality in cultured European sea bass. Fish fed the ARA supplemented feed showed significantly increased concentrations of plasma eicosanoids, compared to broodstock fed northern hemisphere fish oils. While optimum dietary ARA concentration and ARA/EPA ratios are likely to be species-dependent, and influenced by the geography and ecosystem the species inhabits, there is evidence that a high ARA/EPA ratio in eggs may be ubiquitous. Analyses of eggs and larvae from eight species of northern hemisphere marine fish showed that ARA levels were several fold higher than in normal maternal tissue lipids. This suggests that ARA may be specifically concentrated in fish eggs, compared to tissues, and confirms a high biological activity for this HUFA in reproductive processes. In more recent studies, significant improvements in egg and larval quality have been seen in Atlantic halibut broodstock fed diets containing supplemental ARA. Halibut fed diets containing 2% of total fatty acids as ARA showed significant improvements in fertilisation and hatching rates compared to those containing 0.5% or 1.0% ARA (Bell and Sargent 2003). Furuita et al. (2003) found that a supplement of ARA at 0.6 g/100 g diet improved the reproductive performance in broodstock of Japanese flounder *Paralichthys olivaceus*, but a higher level of ARA (1.2 g/100 g diet) negatively affected both egg and larval quality due to a potential inhibitory effect on EPA bioconversion. They also concluded that addition of ARA to broodstock diets improved the egg quality of Japanese flounder. However, excess ARA may have negative effects on reproduction of Japanese flounder. Recent studies suggest the importance of the ratio of EPA to DHA as well as the ratio of n-3 series to n-6 series HUFA in larval and broodstock diets. Efforts should be directed toward establishing the optimum ratio of

DHA/EPA/ARA in diets. More research effort is needed to establish formulated diets appropriate for broodstock of Japanese flounder.

Recently, Hurtado et al. (2009) tested the effect of dietary ARA on reproduction, PGE_2 levels, and some immune variables in oyster *Crassostrea corteziensis*. These results suggest that feeding moderate levels of ARA to oysters can boost immune system response and oocyte production. However, high levels of ARA favor final maturation and advanced stages of vitellogenesis but possibly at the expense of immune response. In addition, he also stated that arachidonic acid is essential for oysters and has been implicated in reproduction and immune response of different mollusk species, probably by modulating the levels of prostaglandins. Arachidonic acid is a precursor for synthesis of eicosanoids, mainly prostaglandins of the series II (PGE_2). ARA is involved in the modulation of the immune system, with both pro-inflammatory and immunosuppressive properties. ARA supplementation in the diet increased heamocyte count, phagocytosis, and production of reactive oxygen species (ROS) by hemocytes in *Crasosstrea gigas* and suggested that ARA directly or its eicosanoid metabolites affect oyster heamocyte functions. During reproduction, there is an inhibition of phagocytic activity and adhesive capacity of hemocytes in *C. gigas*. Immune response can also be a result of increased temperature, which in turn can modulate oocyte maturation, or increase prostaglandin levels. Either way, an increase in PGE_2 has been associated with gonad development and oocyte release during spawning in mollusks. Salze, et. al. 2005 reported that eggs from farmed broodstock had significantly lower levels of ARA, and consequently significantly higher EPA/ARA ratios than eggs from wild broodstock. Therefore, the levels of ARA and phosphatidylinositol, the predominant ARA containing lipid class, and egg pigment content were positively related to egg quality or performance parameters

such as fertilization and hatching success rates, and cell symmetry. Castell et al. (1994) demonstrated that dietary ARA promotes growth of juvenile turbot and concluded that ARA is an essential fatty acid for juvenile turbot. Thus 20:4n-6 may be accumulated by animals consuming a diet relatively high in this fatty acid. Although high levels of 20:4n-6 may not be essential for muscle growth, abalone probably have a requirement for both (n-6) and (n-3) PUFA.



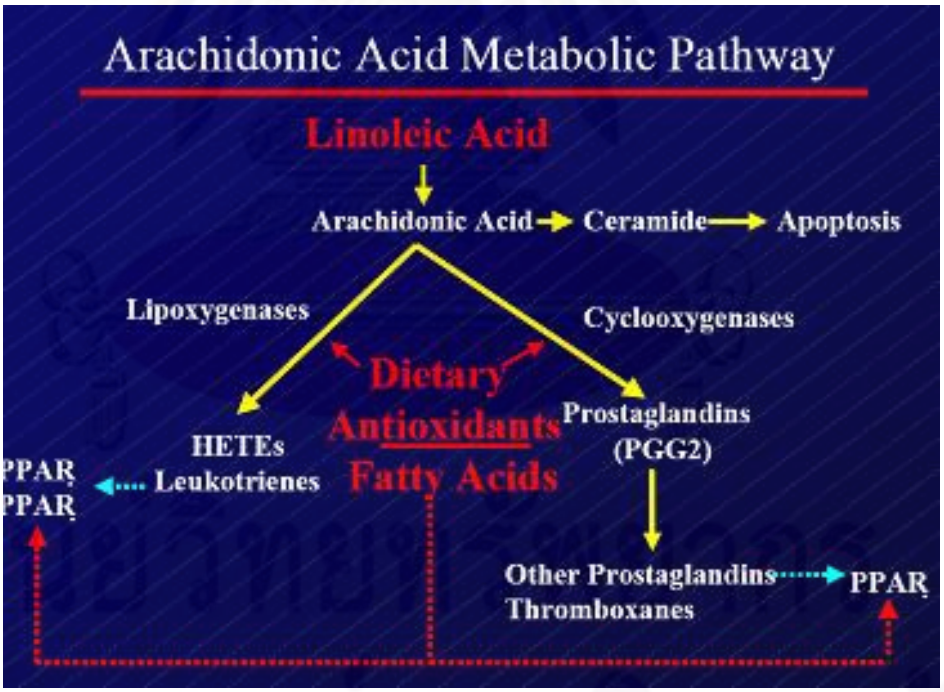


Figure 2-7. Arachidonic acid metabolic pathways (Prescott, 2010 : online and Sanghvi, 2010 : online)

CHAPTER III

REPRODUCTIVE PERFORMANCE, EGG AND LARVAL QUALITY AND EGGS FATTY ACID COMPOSITION OF HATCHERY-REARED SPOTTED BABYLON (*Babylonia areolata*) BROODSTOCK FED NATURAL AND FORMULATED DIETS UNDER HATCHERY CONDITIONS

Introduction

A major constraint to the development of the spotted babylon, *B. areolata*, aquaculture in Thailand is the insufficient supply of seed and high cost production. Successful conditioning of broodstock *B. areolata* is still a crucial step for selective breeding programs to produce a large quantity of eggs and larvae of good quality for the growing industrial importance of this species in Thailand because large variability in spawning events, hatchability, and larval and juvenile survival rates of the spotted babylon has been observed during the same season between batches and hatcheries. This variability remained high despite each batch of larvae being reared in a standardized manner which included the control of larval density, water management and the use of selected microalgal species. Production of good quality larvae is very inconsistent (Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu, 1997). One of the main reasons for unpredictable larvae culture outputs is the variable quality of eggs and larvae. Several factors affect egg and larvae quality in fish and shellfish species. These are either endogenous (genotype, age, and size of broodstock, egg size) or

exogenous (egg management, broodstock feeding, bacterial colonisation of egg surface (Ballestrazzi et al., 2003). In teleosts, nutrients such as protein, fatty acids, vitamin E, ascorbic acids and carotenoids have been implicated in various reproductive-related processes such as gonadal maturation, gamete quality and spawning performances. Interaction between nutrients and reproductive processes, however, remains poorly understood. Several studies have highlighted the importance of both quantity and quality of dietary lipid on reproductive performances of broodstock (Ling et al., 2006). Teruel, Millamena and Fermin (2001) reported that a higher amount of essential nutrients such as protein, lipid and the highly unsaturated fatty acid, e.g. 20:4n-6, 20:5n-3, 22:6n-3 in the artificial diet influenced the increased reproductive performance for abalone, *Haliotis asinina*. Utting and Millican (1997) reported that the number of eggs produced and polyunsaturated fatty acid composition of the eggs of marine bivalves (scallops, oysters and clams) are influenced by the quantity and quality of lipid in microalgae diet supplements. Under optimal hatchery rearing conditions, differences in initial egg lipid reserves may not necessary affect subsequent larval growth and survival. In addition, the importance of lipid and PUFA reserves, in particular, eicosapentaenoic acid, during the development of embryos and larvae can, however, be clearly demonstrated under more stressful rearing conditions. It remains unclear which constituents are responsible for triggering maturation, egg laying of broodstock, therefore, more detailed research on reproductive performance is needed. There are no published studies on the influence of nutrition on the reproductive performance of spotted babylon broodstock, despite their importance in commercial aquaculture. Thus, there is a need to develop a reliable technique for spotted babylon broodstock development through dietary manipulation. This study aimed to determine the effects of dietary lipid sources and concentrations on

reproductive performance, the egg and larval quality and egg fatty acid composition in spotted babylon as a guideline for development of appropriate practical diets for broodstock of this species.

Materials and methods

Study site

This experiment was carried out at the hatchery of the Research Unit and Technology Transfer for Commercial Aquaculture of the Spotted Babylon, Aquatic Resources Research Institute, Chulalongkorn University, Petchaburi Province.

Duration

This experiment was carried out during March to June 2008 due to this period is the high peak of spawning season for this species (Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu, 1997). The feeding trials were conducted for 120 days.

Experimental diets

Four practical diets were formulated to contain different levels of tuna oil and soybean oil based on the ingredient composition outlined in Table 3-1. Treatments were arranged in a completely randomized experimental design. The natural food of fresh meat of carangid fish (*Selaroides leptolepis*) was used as control diet (Figure 3-1a). Experimental diets were formulated with 5 and 15% lipids originated from tuna oil (TO) and mixed oils (MO) (3:2 w/w of tuna oil and soybean oil) and four dietary treatments were labeled as 5%TO, 15%TO, 5%MO and 15%MO respectively. The diets were prepared by weighing the dry ingredients and mixing thoroughly in a

mixer. The lipid sources were added drop by drop while the mixture was further blended to ensure homogeneity. Approximately 200 ml warm water was then added for each kg of this mixture. The diets were extruded and dried using electric fan at room temperature for 12 h. All experimental diets were then stored at -20^{0}C until use. The proximate compositions and major fatty acid composition of the experimental diets were analyzed according to standard methods (AOAC, 1990). While feeding, the feeds were formed into small pieces of 1.5-inch diameter to facilitate sucking by the snails (Figure 3-1b). Uneaten diets in each tank were removed immediately to prevent contamination of seawater. Spotted babylon broodstock were initially fed fresh meat of the carangid fish, *Selaroides leptolepis*, and gradually switched to the experimental diets by the second week of culture. The broodstock were fed the experimental diet once daily at 10:00 hours with the daily amount calculated as 15% of total broodstock biomass per tank. Excess diet was removed and the feeding rate was adjusted based on weight gain after each sampling, which was done every 2 weeks. Mortalities were recorded daily. The feeding trials were conducted for 120 days.

Broodstock sources and acclimation

This experiment was carried out during the spawning season from March to June 2008 (Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu, 1997). Female and male, *B. areolat*a, broodstock used in this study were already used in the commercial private hatchery for 4-6 months. They were transferred to the hatchery of the Research Unit for Commercial Aquaculture of the Spotted Babylon, Aquatic Resources Research Institute, Chulalongkorn University, Petchaburi Province, Thailand. During a 30-day acclimation period, broodstock were held in rearing tanks (3.0x5.0x0.5 m) supplied with flow-through system of natural seawater with moderate aeration. Broodstock

were fed with the conventional food (traditional trash fish) twice daily. At the beginning of the experiment, the females and males were identified and graded to the same size with an average individual wet weight and total length of 46.5–50.3 g and 5.5-7.5 cm, respectively.

Broodstock rearing system

Three hundred broodstock were randomly distributed with a female:male ratio of 10:10 into 15 units. Each plastic tank was 0.5 m x 1.5 m x 0.5 m, with three replicate tanks per dietary treatment. The tank bottoms were covered with a 5 cm layer of coarse sand as substratum for burying of the broodstock. Unfiltered natural seawater was supplied in a flow-through system at a constant flow rate of 5 l/min for 6 h daily and adequate aeration was provided throughout the experimental period. A constant water depth of 30 cm was maintained. Feeding was carried out by hand to apparent visual satiety at 10:00 hours. Sufficient food as could be consumed by the snails was provided over 60 min. To prevent degradation of the seawater, uneaten diets in each tank were removed immediately after the snails stopped eating. Tanks and sand substrate were cleaned of faeces at 15 day intervals by flushing it with a jet of water. Thereafter, the tanks were refilled with new ambient natural seawater. Water temperature, salinity, dissolved oxygen, nitrite nitrogen and ammonia nitrogen during feeding experiment ranged between 30.0 - 32.0^{0}C, 29 - 30 ppt, 4.5 - 7.0 mg L^{-1}, 0 - 0.17 mg L^{-1} and 0 - 0.04 mg L^{-1}, respectively. The rearing tanks were kept under a natural photoperiod. The spotted babylon broodstock were checked for spawning each day in the early morning.

Reproductive performance

Reproductive performance was expressed in terms of total number of spawning, monthly spawning frequency, number of eggs/embryos per capsules, total egg capsule production, total egg/embryo production, sizes of egg capsules, incubation time and hatching rate. Egg capsules produced naturally by female broodstock given each experimental diet were collected every day during the experimental period of 3 months.

For each spawn, egg capsules were collected from each tank by gently scooping them with a net or by hand collection. The number of spawning animals and number of egg capsules spawned were recorded for each feeding trial, thereafter, the total number of egg capsules produced and monthly spawning frequency (average spawning number per month) were estimated at 30 day intervals. The total mean egg production was estimated from total egg capsule production throughout the experimental period multiplied by the average number of eggs/embryos per capsule.

Egg quality

Egg quality was expressed in terms of length and width of egg capsules, number of fertilized eggs per egg capsule, diameter of fertilized eggs and hatching rate. For each spawn, thirty egg capsules were sampled from each tank and measured (length and width) and the number of fertilized eggs/embryos within each egg capsule were counted. Thereafter, the diameter of 20 fertilized eggs in 30 egg capsules from each spawn was measured under an inverted microscope at x 400 magnification and averaged for each batch. To determine the hatching rate, egg capsules of each batch were placed in separate hatching jars of 1 L capacity. All jars were set up with low water flow and low aeration. The water was turned off ca. 2 h before hatching began.

The hatching duration of each batch was recorded. The hatching rate of eggs (expressed as percentage) was determined by counting the number of unhatched eggs in three 1 ml samples, calculating the total of unhatched eggs, and subtracting these from the total number of successfully fertilized eggs.

Larval quality

Larval quality was expressed in terms of the initial shell length of newly hatched larvae, starvation tolerance test and final shell length at the end of starvation tests. The quality of larvae was determined by observing their phototaxic response. After switching off aeration, weak and dead larvae concentrated at the bottom of the tank were siphoned out and triplicate samples were counted. The newly-hatched larvae from each spawn were sampled (n = 50) and the initial shell length (SL) was measured microscopically.

Starvation tolerance tests were conducted with larvae to check the quality of larvae in the stress condition of no food supply. From each batch, three replicate groups of 100 larvae were placed in 2-L plastic containers in order to detect the time of 100% mortality under starvation conditions and standardized larvae culture methods at 30 ± 1^{0}C and 29 ± 1 ppt (Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu, 1997). The starvation period was recorded at 100% mortality and final shell length of larvae were measured (n = 30).

Biochemical composition of the egg capsules

At the end of the experiment, 200 egg capsules from each replicate tank (n=3) were pooled, and stored frozen at -20^{0}C for subsequent biochemical analysis. All samples were analyzed at the Laboratory Center for Food and Agricultural Product

(LCFA), Bangkok, Thailand. Egg capsules from each dietary treatment were analyzed for proximate analysis (crude protein, total fat, carbohydrate, ash and moisture) according to standard methods (AOAC, 1990). Fatty acid determination in experimental diets and egg capsules was performed by gas-liquid chromatography (GLC) based on AOAC (1990).

Briefly, the total lipid was first extracted from samples of each diet. An aliquot of the liquid extract obtained was separated by homogenization in chloroform/methanol (2:1, v/v), methylated and transesterified with boron trifluoride in methanol. Fatty acid methyl esters (FAME) were separated and quantified by using gas-liquid chromatography (Automatic System XL, Perkin Elmer) equipped with a flame ionization detector (FID) and a 30 m x 0.25 mm fused silica capillary column (Omegawax 250, Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas and temperature tprogramming was from $50C^0$ to 220^0C at 4C/min, and then held at 220^0C for 35 min. The injector and detector temperatures were 250^0C and 260^0C respectively. Individual FAME was identified by comparing their retention times with those of authentic standards (Sigma Chemical Company, St. Louis, Missouri, USA).

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). The statistical significance of differences among treatments was determined using one-way analysis of variance (ANOVA), and Duncan's multiple range test ($p<0.05$) was applied to detect significant differences between means ($p<0.05$).

Table 3-1. Experimental formulated diets for *B. areolata* broodstock containing different sources and levels of dietary lipids

Ingredients (% diet)	Control[a]	5%TO[a]	15%TO[a]	5%MO[a]	15%MO[a]
Fish meal	-	20.0	20.0	20.0	20.0
Shrimp meal	-	20.0	20.0	20.0	20.0
Squid meal	-	10.0	10.0	10.0	10.0
Soybean meal	-	20.0	20.0	20.0	20.0
Tuna oil	-	5.0	15.0	-	-
Mixed tuna oil and soybean oil	-	-	-	5.0	15.0
Wheat flour	-	8.0	8.0	8.0	8.0
Cellulose		10.0		10	
Polymethylocarbamide	-	3.0	3.0	3.0	3.0
Wheat gluten		1.5	1.5	1.5	1.5
Vitamin mix[1]	-	1.0	1.0	1.0	1.0
Mineral mix[2]	-	1.0	1.0	1.0	1.0
Chlolesteral		0.5	0.5	0.5	0.5
Proximate composition (%)					
Crude Protein	19.81±0.01	36.24±0.1	35.93±0.6	37.71±0.3	37.61±0.1
Total fat	1.31±0.01	18.64±0.05	26.54±0.1	16.76±0.3	25.37±0.3
Carbohydrate	nd.	19.75±0.4	12.78±0.4	20.49±0.6	11.47±0.4
Moisture	77.6±0.01	8.17±0.2	7.68±0.8	8.41±0.2	8.52±0.3
Ash	1.31±0.03	12.21±0.3	12.01±1.2	11.96±0.7	11.83±0.1

[1] Vitamins (% kg^{-1} diet): vitamin A 107 IU, vitamin D 106 IU, vitamin E 0.01%, vitamin K 0.001%, vitamin B_1 0.0005%, vitamin B_6 0.01%, Methionin 0.016%.

[2] Minerals (% kg^{-1} diet): dicalcium phosphate 14.7%, phosphorus 14.7%, manganese oxde 1.0%, copper sulphate 0.36%, iron sulphate 0.20%, potassium iodide 0.10%, cobalt sulphate 0.10%, selenium oxide 0.006%

[a] Diet abbreviations are as follows: Control = fresh meat of carangid fish (*Selaroides leptolepis*); TO = tuna oil; MO = mixture of tuna oil and soybean oil (3:2).

nd. = not detect

(a)

(b)

Figure 3-1. Fresh meat of carangid fish; *(Selaroides leptolepis*) used as control food (a) and formulated experimental diets molded in round shape (b)

Results

Biochemical composition of experimental diets

The proximate composition and fatty acid composition of the experimental formulated diets are presented in Table 3-2. The levels of protein content did not differ significantly among the different experimental diets and ranged 35.93% to 37.71%, but the lipid content of diets of 15%TO (26.54%) and 15%MO (25.37%) were significantly higher than those of 5%TO (18.64%) and 5%MO (16.76%). Table 3-2 shows the fatty acid composition of the diets. All formulated diets contained higher total unsaturated fatty acids for both monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) than the trash fish. The highest C20:5n -3 (EPA), C22:n6 – 3 (DHA), C20:4n – 6 (ARA), total n – 3 PUFA and n – 3 HUFA were found in the diet of 5%TO. The highest total saturated fatty acids (SFA) were found in the diet of 15%TO, followed by the diets 15%MO, 5%TO, 5%MO and trash fish, respectively.

Broodstock performance

This study was the first attempt to condition *B. areolata* broodstock using formulated diets under hatchery conditions over a period of 120 days. All broodstock groups accepted and readily consumed the experimental formulated diets. They showed no signs of stress as exhibited by active movement and feeding and protrusion of the siphon tube throughout the experiment (Figure 3-2). At the end of the experiment, the mean survival of female broodstock ranged from 80.0% to 95.0% for all dietary treatments. The weight gain of female broodstock increased slightly (0.89 to 1.04 g/snail) and no significant difference was found among dietary treatments

Table 3-2. Fatty acid composition (mg/100g wet weight) of experimental diets

Composition (±SD)	Control[1]	5%TO[1]	15%TO[1]	5%MO[1]	15%MO[1]
C12:0	-	-	7.8±0.8	-	7.1±0.3
C14:0	67.2±0.4^{e}	198.5±0.1^{c}	548.2±0.1^{a}	146.7±1.0^{d}	349.5±0.9^{b}
C16:0	561.6±0.1^{e}	1,581.3±0.4^{c}	3,898.0±0.1^{a}	1,474.6±0.3^{d}	3,292.2±0.6^{b}
C18:0	289.5±0.5^{d}	525.2±0.2^{c}	1,267.0±0.1^{a}	544.3±0.8^{c}	1,077.5±0.1^{b}
C20:0	23.9±0.04^{e}	26.2±0.3^{d}	81.2±0.1^{a}	30.1±0.1^{c}	74.5±0.5^{b}
C22:0	20.9±0.5^{d}	21.7±0.1^{d}	59.6±0.6^{b}	34.9±0.1^{c}	81.2±0.3^{a}
C24:0	14.9±0.1^{d}	30.7±0.4^{e}	54.1±0.7^{b}	30.2±0.09^{c}	61.4±0.0^{a}
C16:1n7	75.6±0.5^{e}	269.7±0.3^{c}	746.6±0.10^{a}	200.8±1.0^{d}	454.3±0.3^{b}
C18:1n9t	17.3±1.4^{e}	51.2±0.2^{c}	473.1±0.1^{a}	29.6±0.5^{d}	111.9±0.7^{b}
C18:1n9c	50.2±1.3^{e}	814.9±0.8^{d}	1,969.0±0.4^{b}	897.6±0.3^{c}	2,429.5±0.1^{a}
C20:1n11	-	22.2±0.2^{c}	101.8±0.6^{a}	13.8±0.1^{d}	44.4±0.7^{b}
C22:1n9	-	39.3±0.5^{c}	166.1±0.1^{a}	22.9±1.3^{d}	91.8±0.8^{b}
C24:1n9	8.5±0.0^{e}	40.3±0.1^{c}	106.6±0.08^{a}	31.2±0.7^{d}	56.3±0.3^{b}
C18:2n6	10.0±0.2^{e}	403.3±0.2^{d}	477.0±0.7^{c}	1,036.3±0.3^{d}	1,796.7±0.1^{e}
C18:3n3	-	120.3±0.3^{d}	224.1±0.3^{b}	139.1±0.09^{c}	253.2±0.3^{a}
C20:2	-	20.6±0.5^{c}	71.5±0.1^{a}	19.5±0.2^{c}	43.8±0.8^{b}
C20:3n6	-	11.1±0.2	-	-	-
C20:4n6 (ARA)[1]	13.3±0.4^{a}	71.1±0.1^{a}	56.2±0.3^{c}	61.8±0.3^{b}	49.4±0.6^{d}
C20:5n3 (EPA)[1]	6.3±0.3^{e}	99.1±0.1^{a}	34.9±0.7^{d}	59.2±0.1^{b}	38.1±0.2^{c}
C22:6n3 (DHA)[1]	10.9±0.7^{e}	376.4±1.3^{a}	164.8±0.6^{c}	217.7±0.3^{b}	108.0±0.8^{d}
Σ SFA[1]	978.2±0.2^{e}	2,383.7±0.2^{c}	5,915.9±0.5^{a}	2,260.8±0.09^{d}	4,943.4±0.3^{b}
Σ MUFA[1]	151.6±0.1^{e}	1,237.6±0.03^{c}	3,585.4±0.3^{a}	1,195.9±0.3^{d}	3,188.2±0.5^{b}
Σ PUFA[1]	40.5±0.4^{e}	1,101.9±0.2^{c}	1,028.5±0.4^{d}	1,533.6±0.7^{b}	2,291.2±0.1^{a}
Total unsaturated fatty acid	192.1±0.3^{e}	2,339.4±0.1^{d}	4,593.9±0.9^{b}	2,729.5±0.4^{c}	5,479.4±0.3^{a}

Table 3-2. (continued) Fatty acid composition (mg/100g wet weight) of experimental diets

Composition (±SD)	Control[1]	5%TO[1]	15%TO[1]	5%MO[1]	15%MO[1]
Σ n - 3 PUFA	17.2±0.04^{e}	595.7±0.0^{a}	423.8±0.2^{b}	416.0±0.4^{c}	146.1±0.8^{d}
Σ n - 3 HUFA[1]	17.2±0.1^{e}	475.5±0.3^{a}	199.7±0.6^{d}	276.9±0.3^{c}	399.3±0.2^{b}
Σ n - 6 PUFA	23.3±0.3^{e}	485.5±0.3^{d}	533.2±0.1^{c}	1,098.1±0.5^{b}	1,846.1±0.1^{a}
Σ n - 9 PUFA	76.0±0.6^{e}	945.7±0.1^{d}	2,714.8±0.2^{a}	981.3±0.6^{c}	2,689.5±0.8^{b}
DHA / EPA	1.73±0.2^{e}	3.80±0.4^{b}	4.73±0.3^{a}	3.68±0.2^{c}	2.73±0.4^{d}
ARA / EPA	2.10±0.3^{a}	0.78±0.2^{e}	1.61±0.2^{b}	1.04±0.4^{d}	1.30±0.3^{c}

[1] Diet abbreviations are as follows: Control = fresh meat of carangid fish (*Selaroides leptolepis*);

TO = tuna oil; MO = mixture of tuna oil and soybean oil (3:2), SFA = saturated fatty acids,

MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, HUFA = highly unsaturated fatty acids, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid,

ARA = arachinodic acid

Values are means ± SD (n = 3) from three replicate tanks per diet. Means in the same row with different superscript letters are significantly different (p<0.05).

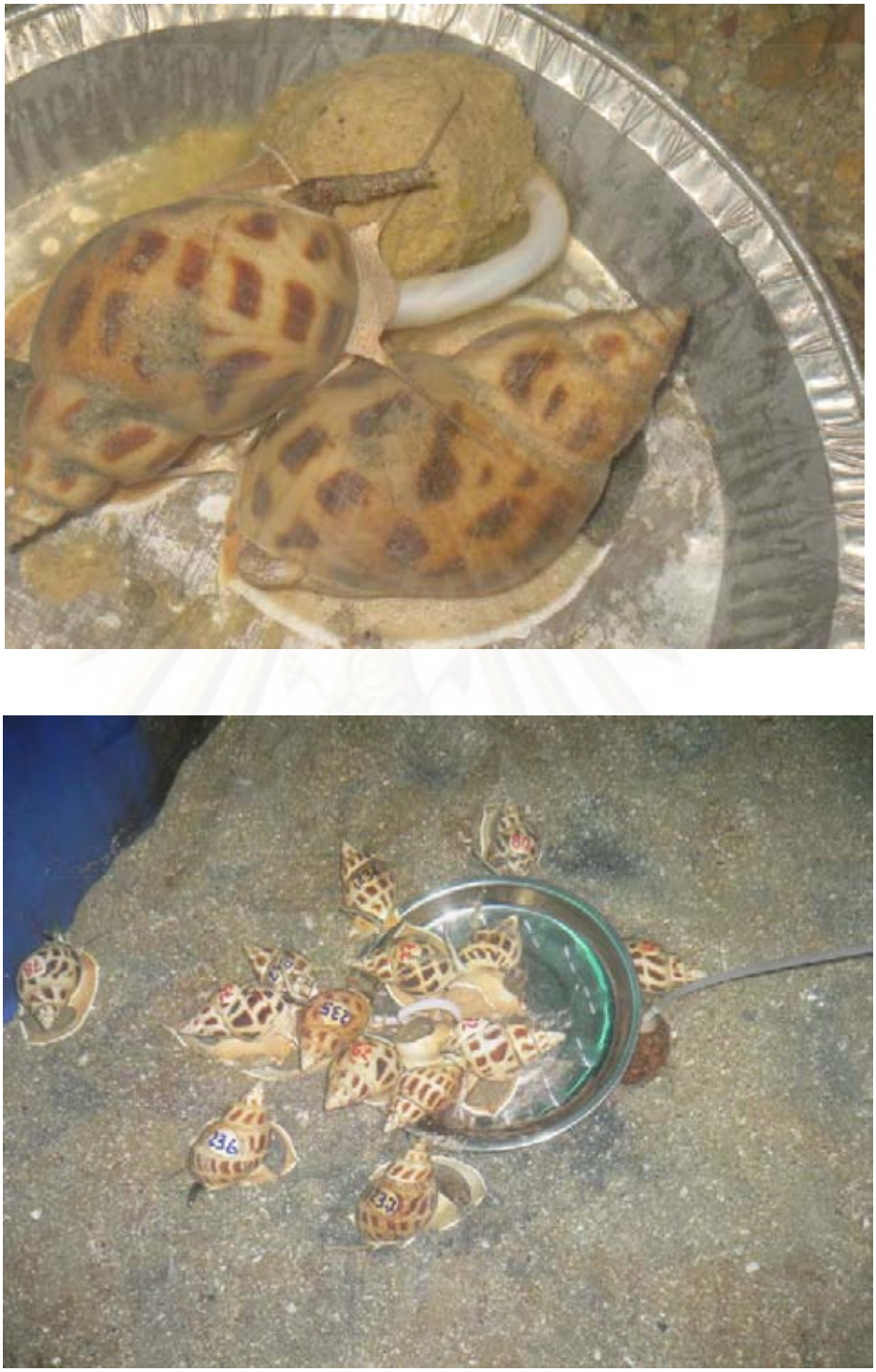


Figure 3-2. Feeding of *B. areolata* broodstock on experimental formulated diets within feeding tray by using proboscis

Reproductive performance

The reproductive performance of the *B. areolata* broodstock fed different experimental diets is presented in Table 3-3. The parameters studied to determine spawning quality (total number of spawning, monthly spawning frequency, total egg capsule production and number of fertilized eggs/embryos per capsule) differ significantly among the broodstocks fed either the experimental formulated diets or the natural food. The mean total number of spawning throughout the experiment differ significantly among dietary treatments ($p<0.05$). Females fed 5%TO diet had the highest total number of spawning (20.6), followed by the control diet (17.6), 5%MO (11.7), 15%TO (8.9) and 15%MO (6.6), respectively. The monthly frequency of spawning for females fed diet 5%TO (5.2 times) was significantly higher ($p<0.05$) than those of females fed control diet (4.4 times), 5%MO (4.4 times), 15%TO (2.7 times) and 15%MO (2.9% times).

Egg quality

The egg quality of the *B. areolata* broodstock fed different experimental diets is presented in Table 3-3. The total egg capsules production obtained from females fed the 5%TO diet (1,985 capsules) and control diet (2,128 capsules, respectively) was significantly higher ($p<0.05$) than those from the females fed the diets of 5%MO (1,021 capsules), 15%TO (861 capsules) and 15%MO (604 capsules). No significant differences in mean number of the fertilised eggs in egg capsules were observed, ranging 375.0 to 415.0 eggs per capsule ($p>0.05$). Total egg production obtained from females fed 5%TO diet (815,739 eggs) and control diet (880,993 eggs) were significantly higher ($p<0.05$) than those from females fed diets with 5%MO diet (383,064 eggs), 15%TO (334,469 eggs) and 15%MO (212,930 eggs) (Table 3-3). The

mean total length and width of egg capsules produced from females fed all experimental diets ranged from 1.71 to 1.80 cm and 0.77 to 0.83 cm, respectively, without significant differences among diets ($p>0.05$).

Larval quality

The larval quality of the sp *B. areolata* broodstock fed different experimental diets are presented in Table 3-3. Eggs needed a minimum of 5 days incubation to start hatching for all dietary treatments, and no remarkable differences were observed in the egg hatching rate (approximately 100%) for all feeding treatments. Likewise, larval quality was not affected by the dietary treatments. Table 3 shows that the initial shell length of the newly-hatched larvae at day 1 ranged 422.0 to 433.0 µm and no significant differences in the initial shell length of the newly-hatched larvae were observed among females fed any of the experimental diets ($p>0.05$).

Time at 100% mortality to starvation tolerance test of larvae produced from the broodstock fed all experimental diets and trash fish were not significantly different, ranging 4.3 to 4.6 days ($p>0.05$). The mean final shell length of larvae at the end of incubation period was not significantly different ($p>0.05$) in any of feeding treatments; it ranged from 431.6 to 442.4 µm.

Table 3-3. Reproductive performance, and egg and larval quality of *B. areolata* broodstock fed on different experimental diets and control diet for 120 days

Parameters	5%TO	15%TO	5%MO	15%MO	Control
1. Broodstock performance					
Initial weight of females (g/snail)	46.51±6.34	46.69±5.87	45.53±3.21	47.50±8.43	50.39±9.50[a]
Final weight of females (g/snail)	47.35±4.82	47.41±4.67	46.57±6.16	48.40±5.93	51.52±10.13[a]
Average weight gain (g/snail)	0.89±0.06[a]	0.92±0.14[a]	0.99±0.08[a]	0.96±0.85[a]	1.03±0.13[a]
Mean survival of females (%)	95.0±7.07[a]	85.0±7.07[a]	85.0±7.07[a]	80.0±14.14[a]	90.0±14.14[a]
2. Reproductive performance					
Total number of spawning	20.6±5.69[a]	8.9±4.59[d]	11.7±5.03[c]	6.6±5.03	17.7±2.52[b]
Monthly frequency of spawning	5.2±2.04[a]	2.7±1.58	4.4±2.44[b]	2.9±2.34[c]	4.4±1.72[b]
Average total egg capsule production	1,985[a]	861[c]	1,021[b]	604[d]	2,128[a]
3. Egg quality					
Mean fertilised eggs / capsule	415±52.19[a]	387±36.11[c]	375±34.3[d]	390±23.4[c]	403±69.03[b]
Length of egg capsules (cm)	1.80±0.07[a]	1.73±0.09[a]	1.75±0.11[a]	1.71±0.21[a]	1.76±0.11[a]
Width of egg capsules (cm)	0.80±0.04[a]	0.78±0.05[a]	0.77±0.08[a]	0.79±0.02[a]	0.83±0.06[a]
Egg incubation time (day)	5.6+0.82[a]	5.2±0.44[a]	5.4±0.52[a]	5.3±0.52[a]	5.4±0.14[a]
Egg hatching rate (%)	100	100	100	100	100
4. Larval quality					
Initial shell length of newly-hatched larvae (µm)	422.00±3.61[a]	424.66±12.14[a]	427.58±6.49[a]	425.16±4.1[a]	433.00±6.55[a]
Time at 100% mortality to starvation test (day)	4.4±0.59[a]	4.5±0.87[a]	4.3±0.58[a]	4.5±0.55[a]	4.6±0.58[a]

Results are means ±S.D. (n = 3)

Control = fresh meat of carangid fish (*Selaroides leptolepis*); TO = Tuna oil; MO = Tuna oil and soybean oil (3:2)

Values in the same row with the same superscript are not statistically different.

No statistical analysis was performed for hatching rate

Chemical composition of the egg capsules

Table 3-4 shows the biochemical composition of egg capsules produced from *B. areoata* broodstock originating from snails exposed to different dietary treatments over 120 days. At the end of the experiment, a total of 13 fatty acids were identified in the egg capsules, with a considerable amount of variation in fatty acid profile within feeding treatments. Protein content in egg capsules produced from the females fed the diet of 15%MO (2.34 g/ 100g) had significantly higher ($p<0.05$) protein content compared to those from the females fed the 5%MO (2.18 g/ 100g), 15%TO (2.05 g/100g), control diet (0.35 g/100 g) and 5%TO (1.90 g/100g). The lipid content of egg capsules produced from the females fed the diet of 15%TO (0.42 g/ 100g) had significantly higher ($p<0.05$) lipid content than other diet groups, ranging from 0.30 to 0.35 g/100g).

Fatty acid compositions of the egg capsules

Table 3-4 shows the fatty acid composition of egg capsules produced from *B. areoata* broodstock originating from snails exposed to different dietary treatments over 120 days. The total saturated fatty acids (SFA) in egg capsules produced from the females fed diet 5%TO (226.6 mg/100 g) was significantly higher ($p<0.05$) than those fed the 15%TO diet (162.9 mg/100g), control diet (159.8 mg/100 g), 15%MO (131.9 mg/100 g) and 5%MO (117.2 mg/100 g). The total unsaturated fatty acids (monounsaturated fatty acid, MUFA and polyunsaturated fatty acid, PUFA) in egg capsules produced from the females fed diet 5%TO (237.7 mg/100 g) was significantly higher ($p<0.05$) than those from the females fed the 15%TO (213.6 mg/100g), 15%MO (171.4 mg/100 g), control diet (149.7 mg/100 g), and 5%MO (145.3 mg/100 g).

Levels of C20:5n-3 (EPA) in egg capsules produced from the females fed the 5%TO diet (48.6 mg/100 g) were significantly higher than those of egg capsules produced from the 15%TO (38.2 mg/100 g), control diet (27.0 mg/100 g), 15%MO diet (19.7 mg/100 g) and 5%MO diet (16.5 mg/100 g), but the levels of C22:6n-3 (DHA)) in egg capsules produced from 15%TO diet (68.2 mg/100 g) were significantly higher than those of egg capsules produced from the 5%TO (54.3 mg/100 g), control diet (49.6 mg/100 g), 15%MO diet (30.0 mg/100 g) and 5%MO diet (25.9 mg/100 g). Similarly, levels of C20:4n-6 (ARA) in egg capsules produced from the females fed the 5%TO (50.9 mg/100 g) were significantly higher compared to those produced from the females fed 15%TO (39.6 mg/100 g), control diet (38.0 mg/100 g), 15%MO (27.1 mg/100 g) and 5%MO diet (21.8 mg/100 g).

The total n-3 PUFA differed significantly between all feeding groups, with the highest total n-3 PUFA level in egg capsules from 5%TO and 15%TO (113.1 and 113.8 mg/100 g, respectively) compared to those from the control diet (76.6 mg/100 g), 15%MO (49.7 mg/100 g) and 5%MO (42.4 mg/100 g). Egg capsules produced from each of the feeding groups differed significantly in the levels of total n–3 HUFA, with the highest total n–3 HUFA level obtained from the 15%TO diets (106.4 mg/100 g), followed by those produced from the 5%TO diet (102.9 mg/100 g), control diet (76.6 mg/100 g), 15%MO (49.7 mg/100 g) and 5%MO (42.4 mg/100 g). Similarly, the total n-6 PUFA in egg capsules produced from the females fed the 5%TO (70.4 mg/100 g) was significantly higher compared to those produced from the females fed 15%MO (68.3 mg/100 g), 5%MO (60.2 mg/100 g), 15%TO (55.2 mg/100 g) and control diet (45.7 mg/100 g).

The ratio of DHA to EPA differed significantly between each broodstock group, with the greatest ratio in egg capsules from the control group, the 15%TO,

5%MO and 15%MO), ranged (1.5:1 to 1.8:1). The ratio of ARA to EPA also differed significantly, with the greatest ratio in egg capsules from the control group, the 5%MO and 15%MO), ranged (1.3:1 to 1.4:1). The (n – 3) /(n – 6) PUFA ratio also differed significantly, with egg capsules from the 15%TO group (2.1:1) having a higher ratio than egg capsules from the control group (1.7:1), 5%TO (1.6:1), 5%MO (0.7:1) and 15%MO (0.7:1).

Table 3-4. Biochemical composition (%) and fatty acid composition (mg fatty acid /100g wet weight) of egg capsules produced from *B. areolata* broodstock fed different experimental diets (n = 3) for 120 days

Egg capsule composition (±SD)	**Control[1]**	**5% TO[1]**	**15% TO[1]**	**5% MO[1]**	**15% MO[1]**
Crude protein	1.93±0.2[a]	1.9±0.4[a]	2.05±1.1[b]	2.18±0.4[c]	2.34±0.6[d]
Total lipid	0.35±0.5[a]	0.31±0.7[a]	0.42±0.1[b]	0.30±1.0[a]	0.34±0.2[a]
Saturated fatty acids (SFA)					
C14:0	*	6.1±0.5	*	*	*
C16:0	87.7±0.2[c]	129.1±0.1[a]	95.6±0.3[b]	70.1±0.3[e]	77.9±0.1[d]
C18:0	51.8±0.1[c]	74.2±0.5[a]	54.7±0.1[b]	38.4±0.2[e]	44.3±0[d]
C24:0	9.4±0.1	*	*	*	*
Monounsaturated fatty acids (MUFA)					
C18:1n9c	19.4±0.08[d]	33.9±1.2[b]	30.0±0.6[c]	30.6±1.3[c]	34.2±1.1[a]
C20:1n11	8.1±0.3[d]	14.1±0.0[a]	14.6±0.9[a]	12.1±0.1[c]	13.2±0.4[b]
Polyunsaturated fatty acids (PUFA)					
C18:2n6	7.7±0.3[e]	19.5±0.02[c]	15.6±0.6[d]	38.4±0.2[b]	41.2±0.6[a]
C18:3n3	*	10.2±0.7[a]	7.4±0.3[b]	*	*
C20:2	*	6.2±0.8[a]	*	*	6.0±0.2[a]
C20:4n6 (ARA)[1]	38.0±0.2[c]	50.9±0.1[a]	39.6±0.4[b]	21.8±0.3[e]	27.1±0.2[d]

Table 3-4. (continued) Biochemical composition (%) and fatty acid composition (mg fatty acid /100g wet weight) of egg capsules produced from *B. areolata* broodstock fed different experimental diets (n = 3) for 120 days

Egg capsule composition (±SD)	Control[1]	5% TO[1]	15% TO[1]	5% MO[1]	15% MO[1]
C20:5n3 (EPA)[1]	27.0±0.2^{c}	48.6±0.02^{a}	38.2±1.6^{b}	16.5±0.3^{e}	19.7±0.06^{d}
C22:6n3 (DHA)[1]	49.6±0.3^{c}	54.3±0.3^{b}	68.2±0.5^{a}	25.9±1.2^{e}	30.0±0.2^{d}
Σ SFA[1]	148.9±0.1^{c}	209.4±0.3^{a}	150.3±0.2^{b}	108.5±0.1^{e}	122.2±0.1^{d}
Σ MUFA[1]	27.40±0.3^{e}	48.0±0.4^{a}	44.6±0.9^{c}	42.7±0.8^{d}	47.4±0.2^{b}
Σ PUFA[1]	122.3±0.1^{d}	189.7±0.8^{a}	169.0±0.04^{b}	102.6±0.2^{e}	124.0±0.7^{c}
Total unsaturated fatty acid	149.7±0.6^{e}	237.7±0.7^{a}	213.6±0.2^{b}	145.3±0.8^{d}	171.4±0.3^{e}
Saturated / Unsaturated fatty acid	1.07±0.3^{a}	0.95±0.1^{b}	0.76±0.5^{d}	0.81±0.0^{c}	0.77±0.06^{d}
MUFA / PUFA	0.2±0.5^{c}	0.25±0.2^{c}	0.3±0.5^{b}	0.4±0.8^{a}	0.4±0.2^{a}
Σ n - 3 PUFA	76.6±0.2^{b}	113.1±0.3^{a}	113.8±0.1^{a}	42.4±0.5^{c}	49.7±0.2^{c}
Σ n - 3 HUFA[1]	76.6±0.1^{b}	102.9±0.4^{a}	106.4±0.7^{a}	42.4±0.3^{c}	49.7±0.4^{c}
Σ n - 6 PUFA	45.7±0.3^{e}	70.4±0.8^{a}	55.2±0.2^{d}	60.2±0.2^{e}	68.3±0.7^{b}
Σ n - 9 PUFA	19.4±0.08^{d}	33.9±1.2^{b}	30.0±0.6^{c}	30.6±2.3^{c}	34.2±0.1^{a}
(n - 3) / (n – 6) PUFA ratio	1.7±0.4^{b}	1.6±1.0^{b}	2.1±0.4^{a}	0.7±0.2^{c}	0.7±0.7^{c}
DHA / EPA ratio	1.8±0.6^{a}	1.1±0.03^{d}	1.8±0.2^{a}	1.6±0.7^{b}	1.5±0.2^{c}
ARA / EPA ratio	1.4±0.0^{a}	1.0±0.8^{c}	1.0±0.4^{c}	1.3±0.09^{b}	1.4±0.4^{a}

[1] Diet abbreviations are as follows: Control = fresh meat of carangid fish (*Selaroides leptolepis*); TO = tuna oil; MO = mixture of tuna oil and soybean oil (3:2), SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, HUFA = highly unsaturated fatty acids, EPA = eicosapentaenoic acid, DHA = decosahexaenoic acid, ARA = arachinodic acid

Values are means ±SD (n = 3) from three replicate tanks per diet. Means in the same row with different superscript letters are significantly different (p<0.05).

Discussion

The present study indicates that there were statistically significant differences in reproductive performance between the dietary treatments. The reproductive performance in terms of total number of spawning, the monthly frequency of spawning and total egg capsule production obtained from the females fed 5%TO was significantly higher compared to those fed natural diet and the other experimental diets. However, egg quality (sizes of egg capsules, egg incubation time and egg hatching rate) and larval quality (initial shell length of newly-hatched larvae, survival duration at starvation tolerance test and final shell length of larvae) showed no variability among females fed the control diet or any of the experimental diets. The results of our study suggest that the higher reproductive performance of spotted babylon, *B. areolata*, fed the formulated diets would indicate that the nutritional quality of the broodstock diet influences reproduction. Although it is possible to mature and spawn spotted babylon with the natural diet, the provision of an effective formulated diet fed alone can achieve better reproductive performance of the spotted babylon broodstock. This observation could be used to enhance the production of quality seeds for spotted babylon hatcheries. The dietary nutrients, especially in terms of proteins, lipids, and fatty acids, e.g. 20:4n-6 (ARA); 20:5n-3 (EPA), 22:6n-3 (DHA), which are essential in reproduction, and that may be insufficient in the natural diets, may have been compensated by feeding the formulated diets as a supplement or as total food for the spotted babylon. This result agrees with the study of Djunaidah et al. (2003) where artificial diets resulted in a reproductional success of mud crab (*Scylla paramamosain*) comparable to the use of fresh food and the nutritional composition of the artificial diets could be improved in order to produce larvae of

optimal quality. In addition, Teruel et al. (2001) reported that a higher amount of essential nutrients in the artificial diets such as protein, lipid and the highly unsaturated fatty acids, e.g. 20:4n-6, 20:5n-3, 22:6n-3 in hatchery-bred donkey's ear abalone *Haliotis asinina* fed artificial diet alone and a combination of natural diet and artificial diet may have influenced the increased reproductive performance. Further, this result also agrees with the study of Utting and Millican (1997) which showed that, in good environmental conditions, endogenous egg reserves, in particular PUFAs, are important for survival through embryogenesis but not for subsequent larval growth and survival of marine bivalves. Utting and Millican (1998) also demonstrated the important factors for the production and viability of eggs and embryos of scallop (*Pecten maximus*). Essential fatty acids particularly 20:5n-3, 22:6n-3 and 20:4n-6 must be supplied in microalgae diets during broodstock conditioning. *P. Maximus*, like most other bivalves, has limited ability to elongate or desaturate fatty acid precursors and has a dietary requirement for essential polyunsaturated fatty acids , in particular, 20:5n–3, and 22:6n-3. Using unialgal diets deficient in specific fatty acids, it can be shown that the essential fatty acid composition of *P. Maximus* gonad and egg lipids is related to the fatty acids in the microalgae fed to broodstock during hatchery conditioning. In comparison to this study, the formulated diets contain higher levels of 20:5n-3, 22:6n-3 and 20:4n–6 than those fed trash fish and the essential fatty acids , particular 20:5n-3, 22:6n-3 and 20:4n-6, in egg capsules obtained from *B. areolata* broodstock fed formulated diets were higher in those fed trash fish. This clearly demonstrates the dietary origin of these long – chain PUFAs. Utting and Millican (1998) also stated that the hatching success rate of *P. Maximus* is dependent on egg lipid reserves but not for subsequent larval growth rate. Endogenous reserves laid down in the oocyte are utilised by

developing embryos and larvae until exogenous reserves became available as larvae begin to feed. Lipid, protein and carbohydrate reserves supply the energy needed for embryo development. Most of this energy requirement is for shell deposition. The total fatty acid content of egg capsules decreases during the first 5 days of embryonic development and all fatty acids, 20:5n-3 is preferentially utilised during embryogenesis. By contrast, there is no change in the level of 22:6n-3 because this PUFA is conserved and is important for cell membrane structure. However, once larvae have reached the first feeding stage, their subsequent growth, survival and success at metamorphosis is dependent on a very fine balance between both quality and quantity of lipid in the diet provided, especially the 22:6n-3 rather than on the initial oocyte reserves. Growth of larvae is very dependent on sufficient quantities of dietary polar lipids for incorporation into cell membranes as well as of neutral lipids for energy reserves (Delaunay et al., 1992).

Moreover, there have been several studies on broodstock conditioning of egg and larval quality of fish and shellfish with various diets supplemented with fatty acids. Bell and Sargent (2003) suggested that the dietary ARA/EPA/DHA ratio may be a critical factor in diets for broodstock and larvae of various fish and shellfish. Emata et al. (2003) reported that, for the mangrove red snapper, arachinodic acid (ARA) may be nutritionally more important for egg and larvae development and survival and its supplementation in broodstock diets may enhance reproductive performance. Lavens et al. (1999) stated that the nutritional status of the turbot *Scophthalamus maximus* broodstock can affect offspring quality. The acclimation of essential nutrients such as essential fatty acids and vitamin C are dependent on the nutrient reserves in the mother animal, and consequently on the dietary input of broodstock in the period preceding gonadogenesis. In this regard, broodstock nutrition

deserves special attention in order to guarantee optimal survival and development of the larvae during the period of endogenous feeding. It may be even advantageous to start feeding when there might only be a marginal uptake of essential nutrients. However, most of the studies on the essential fatty acids have focused on the qualitative and quantitative requirement of EPA and DHA and their optimum dietary ratio in broodstock and larval diets. Essential fatty acids are one of the nutritional factors which greatly affected egg and larval qualities. Variability in maturation, egg laying, and larval and juvenile survival rates among batches may depend on many factors such as food, environmental factors and genetic background. Moreover, variation in the nutritive composition of the larvae between broods may influence development of larvae in various molluscs (Berntsson et al., 1997, Marasigan and Laureta, 2001; Gallager and Mann, 1986; Soudant et al., 1996; Wilson, Chaparro and Thompson, 1986). Daume and Ryan (2004) reported that there is growing evidence that specific dietary lipids play an important role in gonadogenesis of abalone, *Haliotis fulgens*, and variations of the polyunsaturated fatty acid in the digestive gland and foot tissues over the year coincided with variation in their macroalgal diets. Furthermore, arachidonic acid is an essential fatty acid for the abalone and essential fatty acids are derived from the algal diet and are most likely important in cyclical gonad development.

This study presented that broodstock diets containing different amounts of dietary lipid sources and levels influence reproductive performances and egg fatty acid composition in spotted babylon *B. areolata* but not for egg and larval quality. The data presented here indicate that other factors must be participating thus hampering conclusive detection of the nutritional components determining egg and larval quality in spotted babylon. Furthermore, variability in maturation, egg laying,

and larval and juvenile survival rates among batches may depend on many factors such as food, environmental factors and stresses, uncontrolled genetic. Moreover, variation in nutritive contents of the larvae between broods may arise during gametogenesis and influence the variation in development of larvae in various molluscs. Unpredictable and variable egg quality is a major limiting factor for successful mass production of spotted babylon juveniles. It remains unclear which constituents are responsible for triggering maturation and egg laying of broodstock, therefore, more detailed research on maturation and reproductive performance is needed.

Our preliminary results provide initial evidence that the biochemical compositions of broodstock and egg are influenced by the broodstock diets and these in turn may affect the spawning quality. Further research into hormonal control of *B. areolata* reproduction may help to explain the processes involved as well as the fatty acid composition of egg capsules, hatch-out larvae and quality of larvae. The spotted babylon broodstock will have to be successfully conditioned on farms to secure high egg and larvae quality for advanced and sustainable aquaculture, because only this will enable the optimal selection of breeding programs for further development of this species.

Conclusion

This study was a first preliminary investigation on the impact of different formulated diets and the traditional used trash fish on the quality of spawning, egg and larvae of *B. areolata*. Spawning qualities of broodstock fed on formulated diet appeared to be higher than in broodstock fed with trash fish. This result suggest that the formulated diet used in this study was sufficient to obtain acceptable spawning performances but not for egg and larval quality. However, feed composition and fatty acid requirements of spotted babylon broodstock in particular should be further investigated to better understand the fatty acid requirements of this species.

CHAPTER IV

EFFECT OF ARACHIDONIC ACID LEVELS IN BROODSTOCK DIETS ON SPAWNING PERFORMANCE, EGG AND LARVAL QUALITY AND FATTY ACID COMPOSITION OF EGGS IN SPOTTED BABYLON (*Babylonia areolata*)

Introduction

One of the priorities for spotted babylon, *B. areolata*, farming in Thailand is the development of a fully formulated broodstock diet, which would provide good egg and larval quality but be free of the trash fish components traditionally used by the farmer. Formulated diets are the ideal because their composition is fully controlled by the producer, they are easier to transport and handle on farm and the risks of disease transmission from trash fish components are avoided. The nutritional composition and quality of a broodstock diet is an important contributor to egg and larval quality, since during ovarian development, dietary and maternal reserves are mobilised and transported into the oocytes, where they provided the energy and nutritional requirements for growth and development of the embryo and the yolk sac larvae up till the start of exogenous feeding. Broodstock nutrition also affects reproduction and egg quality of fish. Some feed components are stress to greatly influence spawning quality in several species. Essential fatty acids are one of the nutritional factors, which greatly affect egg and larval quality (Mazorra et al., 2003). Recently, attention has partly shifted to include arachidonic acid, mainly because of its role in eicosanoid

production and the involvement of eicosanoids in a range of physiological functions, including reproduction and egg development. The importance of arachidonic acid in reproduction was first identified in European sea bass broodstock, *Dicentrarchus labrax*, fed diets containing fish oil or a local trash fish, bogue (*Boops boops*). The trash fish diet contained around eightfold more ARA than the fish oil diet and the EPA/ARA ratios were 1.5 and 15 for the trash fish and fish oil diets, respectively. Further studies demonstrated that the broodstock fed the high ARA trash fish diet produced significantly better quality eggs than those fed fish oil and dietary ARA was found to be highly concentrated in the eggs and sperm of sea bass broodstock fed trash fish and in wild-caught broodstock (Bell et al. ,1997). Furuita et al. (2006) showed that the n-6 fatty acid level in eggs was negatively correlated with egg quality parameters in Japanese eel *Anguilla japonica* although eel require both n-3 and n-6 PUFA for optimal growth. This study indicated that the suitable dietary ratio of n-3 and n-6 fatty acids is different between juvenile and broodstock eels. Furuita et al. (2003) also indicated that a supplement of ARA at 0.6 g/100g diet improved the reproductive performance of Japanese flounder *Paralichthys olivaceus*, but a higher level of ARA (1.2 g/100g diet) negatively affected both egg and larval quality due to a potential inhibitory effect on EPA bioconversion. The aim of this study was to assess the spawning performance, egg and larval quality, and fatty acid composition of eggs in pond-reared *B. areolata* broodstock fed formulated diets supplemented with different levels of arachidonic acid.

Materials and methods

Study site

This experiment was carried out at the hatchery of the Research Unit and Technology Transfer for Commercial Aquaculture of the Spotted Babylon, Aquatic Resources Research Institute, Chulalongkorn University, Petchaburi Province.

Duration

This experiment was carried out during February to June 2009 due to this period is the high peak of spawning season for this species (Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu, 1997). The feeding trials were conducted for 120 days.

Experimental diets and feeding

Five dietary treatments were desiged in a completely randomized experimental design. The formulated diets containing 5% tuna oil provided the best results in spawning performance and fatty acid composition of eggs. The diets were prepared by weighing the dry ingredients as shown in Table 4-1 and mixing throughly in a mixer. The commercial synthesized arachidonic acid namely Arabita (Suntory, Osaka Japan) was used as ARA sources in this experiments. Arabita ethyl ester component of 1.89 g contains 240 mg arachidonic acid, 240 mg DHA and 1 mg astaxanthin (Figure 4-1). The lipid sources and four levels of the ethyl esters of ARA (0.4% diet 2, 0.8% diet 3, 1.2% diet 4 and 1.6% diet 5) were added drop by drop while the mixture was further blended to ensure homogeneity. The basic diet without ARA addition was used as control (diet 1). Approximately 200 ml warm water was then added for each kg of this

mixture. The diets were extruded and dried using electric fan at room temperature for 12 h. All experimental diets were then stored at -20^{0}C until use. The proximate compositions and major fatty acid composition of the experimental diets were analyzed according to standard methods (AOAC 1990). While feeding, the feeds were formed into small pieces of 1.5-cm diameter to facilitate sucking by the snails. Uneaten diets in each tank were removed immediately to prevent contamination of seawater. Spotted babylon broodstock were initially fed fresh meat of the carangid fish, *S. leptolepis*, and gradually switched to the experimental diets by the second week of culture. The broodstock were fed the experimental diet once daily at 10:00 hours with the daily amount calculated as 15% of total broodstock biomass per tank. Excess diet was removed and the feeding rate was adjusted based on weight gain after each sampling, which was done every 2 weeks. Mortalities were recorded daily. The feeding trials were conducted for 120 days.

Table 4-1. Experimental formulated diets for *B. areolata* broodstock supplemented with various levels of arachidonic acid

Ingredients (g/100g. diet)	Supplement of ARA levels (%)				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
	0% ARA	**0.4% ARA**	**0.8% ARA**	**1.2% ARA**	**1.6% ARA**
Fish meal	27.0	27.0	27.0	27.0	27.0
Shrimp meal	15.0	15.0	15.0	15.0	15.0
Soybean meal	24.0	24.0	24.0	24.0	24.0
Wheat flour	12.0	12.0	12.0	12.0	12.0
Tuna oil	5.0	5.0	5.0	5.0	5.0
Arabita[1]	-	0.4	0.8	1.2	1.6
Cellulose	10.0	9.6	9.2	8.8	8.4
Carboxymethylcellulose	3.0	3.0	3.0	3.0	3.0
Wheat gluten	1.5	1.5	1.5	1.5	1.5
Vitamin mix[2]	1.0	1.0	1.0	1.0	1.0
Mineral mix[3]	1.0	1.0	1.0	1.0	1.0
Chlolesteral	0.5	0.5	0.5	0.5	0.5
Biochemical compositions					
Crude Protein (% dry matter)	25.73±0.3^{a}	25.47±0.1^{a}	25.96±0.6^{a}	25.92±0.3^{a}	25.51±0.1^{a}
Total fat (% dry matter)	4.97±0.1^{a}	5.22±0.1^{a}	5.5±0.1^{a}	6.02±0.3^{b}	6.43±0.3
n-3 HUFA (mg/100 g diet)	475.5±0.5^{c}	425.7±0.4^{b}	311.7±0.2^{a}	475.6±0.2^{c}	561.1±0.5^{d}
Total ARA (mg/100 g diet)	71.1±0.1^{a}	135.5±0.4^{b}	166.2±0.1^{c}	292.6±0.2^{d}	387.1±0.2^{e}

[1] the commercial synthesized arachidonic acid (Arabita, Suntory, Osaka Japan) which 1.89 g ethyl ester component containing 240 mg arachidonic acid, 240 mg DHA and 1 mg astaxanthin

[2] Vitamins (% kg^{-1} diet): vitamin A 107 IU, vitamin D 106 IU, vitamin E 0.01%, vitamin K 0.001%, vitamin B_1 0.0005%, vitamin B_6 0.01%, Methionin 0.016%.

[3] Minerals (% kg^{-1} diet): dicalcium phosphate 14.7%, phosphorus 14.7%, manganese oxde 1.0%, copper sulphate 0.36%, iron sulphate 0.20%, potassium iodide 0.10%, cobalt sulphate 0.10%, selenium oxide 0.006%

Values are means ±SD (n = 3) from three replicate tanks per diet. Means in the same row with different superscript letters are significantly different (p<0.05).

Figure 4-1. Commercial grade of arachidonic acid namely Arbita (Suntory company, Osaka, Japan) used in this study

Broodstock origin and acclimation

This experiment was carried out during the spawning season from February to May 2009 (Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu, 1997). Pond-reared *B. areolat*a, broodstock used in this study were already used in the commercial private hatchery for 4-6 months and they showed the signs of egg laying and low quality of egg capsules (lower in number, fewer fertilized eggs and smaller sizes of egg capsules) and larvae (high mortality of newly-hatched larvae during the first 5 days after hatching). They were transferred by car about 4 hours to the hatchery of the Research Unit for Commercial Aquaculture of the Spotted babylon, Aquatic Resources Research Institute, Chulalongkorn University, Petchaburi Province, Thailand. During a 30 – day acclimation period, snails were held in rearing tanks of

3.0 m x 5.0 m x 0.5 m supplied with flow-through system and they were fed with fresh trash fish twice daily. At the beginning of the experiment, the females and males were graded to the same size with an individual wet weight of 53.14 – 61.23 g.

Experimental units

Three hundred broodstock were randomly distributed into 15 rearing units (0.5 m x 1.5 m x 0.5 m) at a density of 20 snails per tank (female:male ratio of 1:1) three replicate tanks for each dietary treatment were tested. The tank bottoms were covered with a 5 cm layer of coarse sand as substratum for burying of the broodstock. Unfiltered natural seawater was supplied in a flow-through system at a constant flow rate of 5 l/min for 6 h daily and adequate aeration was provided throughout the experimental period. A constant water depth of 30 cm was maintained. Feeding was carried out by hand to apparent visual satiety at 10:00 hours. Sufficient food as could be consumed by the snails was provided over 60 min. To prevent degradation of the seawater, uneaten diets in each tank were removed immediately after the snails stopped eating. Tanks and sand substrate were cleaned of faeces at 15 day intervals by flushing it with a jet of water. Thereafter, the tanks were refilled with new ambient natural seawater. Water temperature, salinity, dissolved oxygen, nitrite nitrogen and ammonium nitrogen during feeding experiment ranged between 30.0 - 32.0^{0}C, 29 - 30 ppt, 4.5 - 7.0 mg L^{-1}, 0 - 0.17 mg L^{-1} and 0 - 0.04 mg L^{-1}, respectively. The rearing tanks were kept under a natural photoperiod. The spotted babylon broodstock were checked for spawning each day in the early morning.

Spawning performance

Reproductive performance was expressed in terms of total number of spawning, monthly spawning frequency, number of eggs/embryos per capsules, total egg capsule production, total egg/embryo production. Egg capsules produced naturally by female broodstock given each experimental diet were collected every day during the experimental period of 4 months. For each spawn, egg capsules were collected from each tank by gently scooping them with a net or by hand collection. The number of spawning animals and number of egg capsules spawned were recorded for each feeding trial, thereafter, the total number of egg capsules produced and monthly spawning frequency (average spawning number per month) were estimated at 30 day intervals. The total mean egg production was estimated from total egg capsule production throughout the experimental period multiplied by the average number of eggs/embryos per capsule.

Egg quality

Egg quality was expressed in terms of length and width of egg capsules, number of fertilized eggs per egg capsule, diameter of fertilized eggs and hatching rate. For each spawn, thirty egg capsules were sampled from each tank and measured (length and width) and the number of fertilized eggs / embryos within each egg capsule were counted. Thereafter, the diameter of 20 fertilized eggs in 30 egg capsules from each spawn was measured under an inverted microscope at x400 magnification and averaged for each batch. To determine the hatching rate, egg capsules of each batch were placed in separate hatching jars of 1 L capacity. All jars were set up with low water flow and low aeration. The water was turned off ca. 2 h before hatching began. The hatching duration of each batch was recorded. The

hatching rate of eggs (expressed as percentage) was determined by counting the number of unhatched eggs in three 1-mL samples, calculating the total of unhatched eggs, and subtracting these from the total number of successfully fertilized eggs.

Larval quality

Larval quality was expressed in terms of the initial shell length of newly hatched larvae, starvation tolerance test and final shell length at the end of starvation tests. The quality of larvae was determined by observing their phototaxic response. After switching off aeration, weak and dead larvae concentrated at the bottom of the tank were siphoned out and triplicate samples were counted. The newly-hatched larvae from each spawn were sampled (n = 50) and the initial shell length (SL) was measured microscopically.

Starvation stress test

Starvation tolerance tests were conducted with larvae to check the quality of larvae in the stress condition of no food supply. From each batch, three replicate groups of 100 larvae were placed in 1-L plastic beakers in order to detect the time of 100% mortality under starvation conditions and standardized larvae culture methods at 30 ± 1^{0}C and 29 ± 1 ppt (Nilnaj Chaitanawisuti and Sirusa Kritsanapuntu, 1997). The starvation period was recorded at 100% mortality.

Salinity stress test

The low salinity stress test is widely used as a final criterion to evaluate the quality of larvae and juveniles, on the assumption that it will predict further resistant performance to a stress condition during grow-out of fish and shellfish. After 120 day

feeding trials, five groups of 100 larvae each from each feeding trial were directly transferred into low salinity seawater (25 ‰) as a test solution in the 2,000 ml aquaria containing 1 L of sterilized seawater with aeration. Test solution was prepared by blending natural seawater and dechlorinated tap water. Salinity of the test solution was confirmed with a reflectosalinometer. Live larvae were counted after 1 h and percentage survival was calculated for each treatment.

Biochemical composition of the egg capsules

At the end of the experiment, 200 egg capsules from each replicate tank (n=3) were pooled, and stored frozen at -20^{0}C for subsequent biochemical analysis. All samples were analyzed at the Laboratory Center for Food and Agricultural Product (LCFA), Bangkok, Thailand. Egg capsules from each dietary treatment were analyzed for proximate analysis (crude protein, total fat, carbohydrate, ash and moisture) according to standard methods (AOAC, 1990). Fatty acid determination in experimental diets and egg capsules was performed by gas-liquid chromatography (GLC) based on AOAC (1990). Briefly, the total lipid was first extracted from samples of each diet. An aliquot of the liquid extract obtained was separated by homogenization in chloroform/methanol (2:1, v/v), methylated and transesterified with boron trifluoride in methanol. Fatty acid methyl esters (FAME) were separated and quantified by using gas-liquid chromatography (Automatic System XL, Perkin Elmer) equipped with a flame ionization detector (FID) and a 30 m x 0.25 mm fused silica capillary column (Omegawax 250, Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas and temperature programming was from 50C^0 to 220^{0}C at 4C/min, and then held at 220^{0}C for 35 min. The injector and detector temperatures were 250^{0}C and 260^{0}C respectively. Individual FAME was identified by comparing

their retention times with those of authentic standards (Sigma Chemical Company, St. Louis, Missouri, USA).

Statistical analysis

Data are presented as mean ± standard deviation (SD). The statistical significance of differences among treatments was determined using one-way analysis of variance (ANOVA), and Duncan's multiple range test ($p<0.05$) was applied to detect significant differences between means ($p<0.05$).

Results

Biochemical composition of experimental diets

Table 4-1 and 4-2 shows the fatty acid composition of the experimental formulated diets containing different supplementation of arachidonic acid (ARA). No significantly difference in crude protein was not observed among all dietary trials with ranging of 25.47 – 25.96% but crude lipid levels were affected by ARA supplementation. The crude lipid levels in diet 1, 2, 3, 4 and 5 were 4.97%, 5.22%, 5.55%, 6.02%, and 6.43%, respectively. There were significant differences in EPA, DHA, ARA, n-3 HUFA and n-6 PUFA contents among all dietary trials. Diet 5 (1.6% ARA supplementation) showed the highest EPA, DHA, ARA, n-3 HUFA and n-6 PUFA contents among other dietary trials significantly. The total ARA content in diets 1, 2, 3, 4 and 5 were 71.1%, 135.5, 166.2, 292.6 and 387.1 mg/100 g diet, respectively, while those of n-3 HUFA were 475.5, 425.7, 311.7, 475.6 and 561.1 mg/100 g diet, respectively. The ratios of DHA / EPA and ARA / EPA increased with increasing of ARA supplementations. The ARA / EPA ratios of diet 1, 2, 3, 4 and 5 were 0.19, 0.41, 0.69, 0.78 and 0.86, respectively, while those of DHA / EPA ratios were 3.79, 3.49, 3.35, 3.75 and 3.92, respectively.

Table 4-2. Fatty acid composition (mg/100g wet weight) of experimental formulated diets for *B. areolata* broodstock

Composition (±SD)	Supplementation of ARA levels (%)				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
	0% ARA	**0.4% ARA**	**0.8% ARA**	**1.2% ARA**	**1.6% ARA**
C12:0	11.9±0.2^{a}	6.2±0.2^{c}	7.6±0.1^{b}	6.2±0.2^{c}	6.6±0.2^{c}
C14:0	291.1±0.3^{a}	226±0.1^{d}	243.5±0.2^{b}	234.2±0.1^{c}	238.1±0.2^{c}
C16:0	2,136±0.1^{a}	1,537.5±0.5^{d}	1,651.6±0.4^{c}	1,647.9±0.4^{c}	1,670.8±0.4^{b}
C18:0	661.7±0.4^{a}	519.1±0.2^{e}	568.4±0.4^{d}	586.7±0.5^{c}	609.8±0.2^{b}
C20:0	46.1±0.3^{c}	39.2±0.2^{e}	44.7±0.2^{d}	49.7±0.1^{b}	52.3±0.1^{a}
C22:0	43.4±0.2^{d}	43.7±0.4^{d}	60.1±0.5^{c}	78.2±0.4^{b}	90.8±0.5^{a}
C24:0	40.6±0.3^{e}	69.2±0.5^{d}	115±0.4^{c}	155.9±0.5^{b}	188.0±0.5^{a}
C16:1n7	217.9±0.3	286.5±0.4	293.1±0.4	290.1±0.4	292.9±0.4
C18:1n9t	52.5±0.5^{a}	-	10.9±0.1^{c}	-	11.7±0.1^{b}
C18:1n9c	709.8±0.6^{e}	883.7±0.5^{d}	926.4±0.4^{c}	972±0.5^{b}	996.5±0.5^{a}
C20:1n11	51.7±0.3^{e}	66.8±0.5^{d}	75.8±0.2^{c}	85.9±0.5^{b}	92.1±0.5^{a}
C22:1n9	12.7±0.4^{e}	13±0.2^{d}	14.7±0.2^{c}	16.8±0.2^{b}	17.8±0.2^{a}
C24:1n9	38±0.5^{d}	43.3±0.2^{c}	47.8±0.2^{b}	54.5±0.2^{a}	54.0±0.4^{a}
C18:2n6	84.6±0.6^{d}	325.1±0.5^{c}	323.1±0.2^{c}	374.3±0.2^{b}	410.3±0.5^{a}
C18:3n3	10.4±0.4^{e}	58.3±0.5^{c}	52.2±0.4^{d}	60±0.5^{b}	63.5±0.5^{a}
C20:2	-	18.2±0.2^{d}	19.4±0.2^{c}	24.3±0.4^{b}	26.5±0.5^{a}
C20:3n6	5.5±0.3^{e}	17.8±0.2^{d}	23.4±0.2^{c}	37.5±0.5^{b}	44.5±0.5^{a}
C20:4n6 (ARA)	71.1±0.1^{e}	135.5±0.4^{d}	166.2±0.5^{c}	292.6±0.2^{b}	387.1±0.2^{a}
C20:5n3 (EPA)	99.1±0.3^{c}	94.7±0.5^{d}	71.6±0.4^{e}	100.1±0.2^{b}	114.1±0.2^{a}
C22:6n3 (DHA)	376.4±0.4^{b}	331±0.4^{c}	240.1±0.2^{d}	375.5±0.4^{b}	447±0.5^{a}

Table 4-2. (continued) Fatty acid composition (mg/100g wet weight) of experimental formulated diets for *B. areolata* broodstock

Composition (±SD)	Supplementation of ARA levels (%)				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
	0% ARA	**0.4% ARA**	**0.8% ARA**	**1.2% ARA**	**1.6% ARA**
Σ SFA	3,242.5±0.2^{a}	2,449.8±0.4^{d}	2,708.9±0.4^{c}	2,788.4±0.5^{c}	2,896.8±0.2^{b}
Σ MUFA	1,082.6±0.3^{d}	1,293.3±0.2^{c}	1,368.7±0.4^{b}	1,419.3±0.5^{a}	1,464.8±0.5^{a}
Σ PUFA	647.1±0.4^{e}	1,006.6±0.2^{c}	925.8±0.4^{d}	1,304.3±0.2^{b}	1,540.5±0.2^{a}
Total unsaturated fatty acid	1,260.1±0.1^{d}	2,299.9±0.2^{c}	2,294.5±0.5^{c}	2,720±0.4^{b}	3,005.3±0.2^{a}
Σ n - 3 PUFA	485.9±0.3^{c}	484.0±0.2^{c}	363.9±0.4^{d}	535.6±0.2^{b}	624.6±0.2^{a}
Σ n - 3 HUFA	475.5±0.5^{b}	425.7±0.4^{c}	311.7±0.4^{d}	475.6±0.2^{b}	561.1±0.5^{a}
Σ n - 6 PUFA	161.2±0.3^{e}	478.4±0.2^{d}	512.7±0.2^{c}	704.4±0.5^{b}	841.9±0.4^{a}
(n - 3) / (n – 6) PUFA ratio	3.01±0.03^{a}	1.01±0.31^{b}	0.71±0.41^{d}	0.76±0.23^{c}	0.74±0.02^{c}
DHA / EPA ratio	3.79±0.14^{b}	3.49±0.02^{c}	3.35±0.33^{d}	3.75±0.18^{b}	3.92±0.10^{a}
ARA / EPA ratio	0.19±0.32^{e}	0.41±0.08^{d}	0.69±0.12^{c}	0.78±0.40^{b}	0.86±0.16^{a}

Diet abbreviations are as follows: SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, HUFA = highly unsaturated fatty acids, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ARA = arachinodic acid

Values are means ±SD (n = 3) from three replicate tanks per diet. Means in the same row with different superscript letters are significantly different (p<0.05).

Broodstock performance

Growth in weight gain and survival of *B. areolata* broodstock were significantly different among all dietary trials (Table 4-3). Results showed that the body weight gains of broodstock gradually increase for all dietary trials. The highest body weight gain was found in broodstock fed on the diets 5 (0.94 g/snail), followed by diet 4 (0.89 g/snail), diet 3 (0.84 g/snail), diet 2 (0.80 g/snail) and basic diet (0.80 g/snail). While the highest mean survival rate (100%) was found in broodstock fed the basic diet and diet 3, followed by the diet 5 (95.0%), diet 4 (95.0%) and diet 2 (50.0%).

Spawning performance

Table 4-3 shows the reproductive performance of *B. areolata* broodstock fed different experimental diets is supplemented with different levels of arachidonic acid over a period of 120 days. The parameters studied to determine spawning quality (total number of spawning, monthly spawning frequency, total egg capsule production and number of fertilized eggs/embryos per capsule) differ significantly among all dietary trials and these parameters were affected by ARA supplementation. The mean total number of spawning throughout the experiment increased with the increasing supplementation of ARA levels. Females fed on diet 5 had the highest total number of spawning (25.5), followed by the diet 4 (23.0), diet 3 (22.0), basic diet (17.5) and diet 1 (15.5), respectively. The monthly frequency of spawning was also affected by ARA supplementation with an increasing with the increasing levels of ARA supplementation. The highest frequency of spawning was found in group of females fed diet 5 (6.4 per month), followed by those of females fed on diet 4 (5.8 per month), diet 3 (5.5 per month), diet 1 (4.4 per month) and diet 2 (3.9 per month). However, the

average production of egg capsule per month and total egg capsule production did not increase with increasing of ARA supplementations. The average production of egg capsule per month was the highest for females fed on diet 3 (766), followed by those fed on diet 6 (619), diet 1 (609), diet 2 (588) and diet 3 (565), while the highest total egg capsule production was found in group of females fed diet 3 (3,064 capsules), followed by those of females fed on diet 5 (2,477 capsules), diet 1 (2,437 capsules), diet 1 (2,353 capsules) and diet 4 (2,262 capsules).

Egg quality

The egg quality (number of fertilized eggs/embryos per capsule, length of egg capsule, egg incubation duration and hatching rate) of *B. areolata* broodstock fed different experimental diets is presented in Table 4-3. Significant differences in mean number of the fertilised eggs/embryos per egg capsule were observed, ranging 563 to 659 eggs per capsule ($p<0.05$). The mean total length and width of egg capsules produced from females fed all experimental diets ranged from 1.91 to 2.14 cm and 0.85 to 0.94 cm, respectively, without significant differences among diets ($p>0.05$). The mean egg incubation time ranged from 5.0 – 5.25 days, without significant differences among diets ($p>0.05$), while the hatching rate was 100% for all dietary treatments.

Larval quality

The larval quality (length of newly hatched larvae and tolerance of larvae to low salinity stress and starvation test) of *B. areolata* broodstock fed different experimental diets is presented in Table 4-3. The highest shell length of the newly - hatched larvae was obtained from the female fed on diet 1 (540 microns) and

diet 2 (548 microns) compared to those fed on diet 3 (527 microns), 4 (538 microns) and 5 (535 microns) with significant difference among dietary trials ($p<0.05$). ARA did not enhance tolerance to low salinity stress and starvation test together with increasing the levels of dietary ARA in *B. areolata* broodstock. All dietary trials had no significantly differences in durations at 100% mortality of starvation stress test and low salinity stress test with ranging from 5.9 – 6.2 days and 5.5 – 6.0 days, respectively ($p>0.05$).

Table 4-3. Reproductive performance and egg and larval quality of *B. areolata* broodstock fed different experimental diets for 120 days

Parameters	**Supplementation of ARA levels (%)**				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
	0% ARA	**0.4% ARA**	**0.8% ARA**	**1.2% ARA**	**1.6% ARA**
1. Broodstock performance					
Initial weight of females (g/snail)	52.61±1.02	53.45±4.73	53.14±2.04	56.40±0.26	64.78±0.60
Final weight of females (g/snail)	53.38±0.33	54.25±1.91	53.97±2.38	57.29±0.36	65.72±3.79
Average weight gain (g/snail)	0.77±0.69	0.80±2.23	0.84±0.33	0.89±0.55	0.94±3.26[a]
Mean survival of females (%)	100[a]	50.0±42.43[c]	100[a]	95.0±7.07[b]	95.0±2.21[b]
2. Reproductive performance					
Total number of spawning (times)	17.5±0.71[d]	15.5±2.12[e]	22.0±11.31[c]	23.0±0.71[b]	25.5±0.71[a]
Frequency of spawning (times / month)	4.4±0.09[d]	3.9±0.27[e]	5.5±1.41[c]	5.8±0.09[b]	6.4±0.09[a]
Total egg capsule production (capsules)	2,437±12.8[b]	2,353±14.12[c]	3,064±15.19[a]	2,262±9.82[d]	2,477±9.72[b]

Table 4-3. (continued) Reproductive performance and egg and larval quality of *B. areolata* broodstock fed different experimental diets for 120 days

Parameters	Supplementation of ARA levels (%)				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
	0% ARA	**0.4% ARA**	**0.8% ARA**	**1.2% ARA**	**1.6% ARA**
3. Egg quality					
Mean embryos per capsule	655±42.76	563±72.37	609±142.93	659±105.94[a]	653±149.83
Length of egg capsules (cm)	1.99±0.08	1.91±0.06	2.03±0.11	2.01±0.09	2.14±0.21[a]
Width of egg capsules (cm)	0.85±0.06	0.88±0.05	0.93±0.06	0.94±0.02[a]	0.91±0.03
Egg incubation time (day)	5.25+0.5	5.0±0.82	5.25±0.5	5.25±0.5	5.25±0.5
Egg hatching rate (%)	100	100	100	100	100
4. Larval quality					
Shell length of newly-hatched larvae (µm)	548.50±3.91[a]	540.88±13.52	527.11±13.16	532.18±27.53	535.95±14.56
Time at 100% mortality to starvation test (day)	5.91±0.8	5.78±0.56	6.21±0.87[a]	6.14±0.45	6.1±0.64
Time at 100% mortality to low salinity test (day)	5.5±0.55	6.0±0	6.0±0	6.0±0	6.0±0

Values are means ±SD (n = 3) from three replicate tanks per diet. Means in the same row with different superscript letters are significantly different ($p<0.05$).

Diet abbreviations are as follows: ARA = arachinodic acid

Fatty acid compositions of the egg capsules

Table 4-4 shows the fatty acid composition of egg capsules produced from *B. areoata* broodstock fed formulated diets with different levels of ARA supplementations over 120 days. At the end of the experiment, a total of 13 fatty acids were identified in the egg capsules, with a considerable amount of variation in fatty acid profile within feeding treatments. Significant differences in fatty acid compositions were found among all dietary trials ($p<0.05$).

The C20:5n-3 (EPA), C22:6n-3 (DHA) and C20:4n-6 (ARA) differed significantly between all feeding groups. The C20:5n-3 in egg capsules produced from the females fed on diet 1 (60.0 mg/100 g diet) was significantly higher than those of egg capsules produced from the diet 5(43.9 mg/100 g diet), diet 3 (37.2 mg/100 g diet), diet 4 (19.7 mg/100 g diet) and diet 2 (16.0 mg/100 g diet). Similarly, the C22:6n-3 in egg capsules produced from the females fed on diet 1 (54.8 mg/100 g diet) was significantly higher than those of egg capsules produced from the diet 5 (41.3 mg/100 g diet), diet 3 (33.7 mg/100 g diet), diet 4 (20.2 mg/100 g diet) and diet 2 (12.4 mg/100 g diet). However, levels of C20:4n-6 in egg capsules produced from the females fed on diet 5 (48.3 mg/100 g diet) was significantly higher than those of egg capsules produced from the diet 1 (43.3 mg/100 g diet), diet 3 (45.9 mg/100 g diet), diet 4 (31.0 mg/100 g diet) and diet 2 (20.1 mg/100 g diet).

The total n-3 PUFA differed significantly between all feeding groups, with the highest total n-3 PUFA level in egg capsules from the females fed on diet 1 (140.6 mg/100 g diet) was significantly higher than those of egg capsules produced from the diet 5 (114.0 mg/100 g diet), diet 3 (104.0 mg/100 g diet), diet 4 (68.6 mg/100 g diet) and diet 2 (46.9 mg/100 g diet). Similarly the egg capsules produced from each of the feeding groups also differed significantly in the levels of total n-3 HUFA, with the

highest total n-3 HUFA level obtained from the females fed on diet 1 (114.8 mg/100 g diet) was significantly higher than those of egg capsules produced from the diet 5 (85.2 mg/100 g diet), diet 3 (70.9 mg/100 g diet), diet 4 (39.9 mg/100 g diet) and diet 2 (28.4 mg/100 g diet). However, the total n-6 PUFA in egg capsules produced from the females fed the diet 3 (83.4 mg/100 g diet) was significantly higher compared to those produced from the females fed on diet 5 (79.2 mg/100 g diet) diet 1 (74.5 mg/100 g diet), diet 4 (61.1 mg/100 g diet) and diet 2 (40.3 mg/100 g diet).

The ratio of DHA to EPA differed significantly between each broodstock group, with the greatest ratio in egg capsules produced from the females fed the diet 4 (1.03:1), followed by those fed on diet 5 (0.94:1), diet 1 (0.91:1) and diet 3 (0.91:1) and diet 2 (0.78:1). While the ratio of ARA to EPA also differed significantly, with the greatest ratio in egg capsules produced from the females fed on diet 4 (1.57:1), followed by those fed on diet 2 (1.25:1), diet 3 (1.23:1), diet 5 (1.1:1) and diet 1 (0.72:1). The (n – 3) /(n – 6) PUFA ratio also differed significantly, with egg capsules produced from the females fed on diet 1 (1.89:1) compared to those fed on diet 5 (1.44:1), diet 3 (1.25:1), diet 2 (1.16:1) and diet 4 (1.11:1).

Table 4-4. Biochemical composition (%) and fatty acid composition (mg fatty acid /100g wet weight) of egg capsules produced from *B. areolata* broodstock fed different experimental diets (n = 3) for 120 days

Parameters	Supplementation of ARA levels (%)				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
	0% ARA	**0.4% ARA**	**0.8% ARA**	**1.2% ARA**	**1.6% ARA**
Egg capsule composition					
Crude protein	2.74±0.5	3.01±0.3	2.91±0.3	2.93±0.4	3.04±0.2
Total lipid	0.60±0.7	0.38±0.5	0.67±0.3	0.48±0.4	0.69±0.3
Saturated fatty acids					
C14:0	7.7±0.3	6±0.2	9.7±0.3	8.2±0.2	7.5±0.1
C16:0	180±0.2	162.8±0.2	244.1±0.1	237.2±0.3	208.3±0.2
C18:0	97.1±0.3	94.2±0.1	146.1±0.3	129.1±0.2	112.9±0.3
C24:0	6.8±0.2	6.2±0.1	5.2±0.3	8.7±0.2	8.4±0.2
Monounsaturated fatty acids					
C18:1n9c	52.6±0.6	41.1±0.2	64.5±0.3	60.6±0.4	53.2±0.2
C20:1n11	46.6±0.3	37.5±0.1	58.1±0.2	53.1±0.2	49±0.2
Polyunsaturated fatty acids					
C18:2n6	31.2±0.4	20.2±0.5	37.5±0.3	30.6±1.0	30.9±0.6
C18:3n3	25.8±0.5	18.5±0.7	33.1±0.8	28.7±0.2	28.8±0.2
C20:2	12±0.3	8.8±0.2	15.5±0.4	10±0.3	11.4±0.2
C20:4n6 (ARA)	$43.3±0.6^{c}$	$20.1±0.7^{a}$	$45.9±0.6^{d}$	$31±0.3^{b}$	$48.3±0.2^{e}$
C20:5n3 (EPA)	$60.0±0.3^{e}$	$16±0.8^{a}$	$37.2±0.5^{c}$	$19.7±0.8^{b}$	$43.9±0.6^{d}$
C22:6n3 (DHA)	$54.8±0.2^{e}$	$12.4±0.7^{a}$	$33.7±0.6^{c}$	$20.2±0.7^{b}$	$41.3±0.5^{d}$
Σ SFA	$358±0.3^{b}$	$320±0.5^{a}$	$490.9±0.7^{e}$	$460.9±0.8^{d}$	$410.5±0.6^{c}$
Σ MUFA	$110±0.2^{b}$	$87.0±0.3^{a}$	$142.1±0.2^{d}$	$129.2±0.4^{c}$	$112.5±0.5^{b}$
Σ PUFA	$227.2±0.3^{d}$	$96±0.5^{a}$	$202.9±0.3^{c}$	$140.2±0.2^{b}$	$204.6±0.1^{c}$

Table 4-4. (continued) Biochemical composition and fatty acid composition (mg fatty acid /100g wet weight) of egg capsules produced from *B. areolata* broodstock fed different experimental diets (n = 3) for 120 days

Parameters	Supplementation of ARA levels (%)				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
	0% ARA	**0.4% ARA**	**0.8% ARA**	**1.2% ARA**	**1.6% ARA**
Total unsaturated fatty acid	337.2±0.4^{d}	183±0.3^{a}	345±0.3^{e}	269.4±0.2^{c}	320.2±0.4^{b}
Saturated / Unsaturated fatty acid	1.06±0.1^{a}	1.75±0.2^{d}	1.42±0.2^{c}	1.71±0.4^{d}	1.28±0.09^{b}
Σ n - 3 PUFA	140.6±0.3^{e}	46.9±0.3^{a}	104±0.4^{c}	68.6±0.2^{b}	114±0.2^{d}
Σ n - 3 HUFA	114.8±0.2^{e}	28.4±0.5^{a}	70.9±0.3^{c}	39.9±0.3^{b}	85.2±0.3^{d}
Σ n - 6 PUFA	74.5±0.3^{c}	40.3±0.7^{a}	83.4±0.2^{e}	61.6 ±0.3^{b}	79.2±0.1^{d}
(n - 3) / (n – 6) PUFA ratio	1.89±0.2^{d}	1.16±0.2^{a}	1.25±0.4^{b}	1.11±0.2^{a}	1.44±0.1^{c}
DHA / EPA ratio	0.91±0.5^{b}	0.78±0.1^{a}	0.91±0.2^{b}	1.03±0.3^{c}	0.94±0.2^{b}
ARA / EPA ratio	0.72±0.2^{d}	1.25±0.4^{b}	1.23±0.4^{b}	1.57±0.4^{c}	1.1±0.6^{a}

Diet abbreviations are as follows: SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, HUFA = highly unsaturated fatty acids, EPA = eicosapentaenoic acid, DHA = decosahexaenoic acid, ARA = arachinodic acid

Values are means ±SD (n = 3) from three replicate tanks per diet. Means in the same row with different superscript letters are significantly different (p<0.05).

Discussion

The present study showed that the parameters studied to determine spawning quality (total number of spawning and monthly spawning frequency) were affected by ARA supplementation but not for total number of egg capsule production. The mean total number of spawning, monthly spawning frequency throughout the experiment increased with the increasing supplementation of ARA levels. Females fed on diet 5 had the highest total number of spawning and frequency of spawning. While the egg quality (number of fertilized eggs in capsule, length and width of egg capsules, egg incubation time and hatching rate) and larval quality (length of newly-hatched larvae and survival of larvae under starvation stress test and low salinity stress test) was not affected by ARA supplementation. This study indicated that the spawning performance increased with the increasing of ARA supplementation and the broodstock fed diet 5 containing highest n-3 HUFA and arachidonic acid had the best spawning performance among all dietary trials. This study showed the same trends of previous studies in fish and shellfish; oyster *Crasosstrea gigas* (Hurtado et al., 2009), Japanese flounder *Paralichthys olivaceus* (Furuita et al., 2003), Japanese eel broodstock *Anguilla japonica* (Furuita et al., 2007). Hurtado et al. (2009) suggested that feeding moderate levels of ARA to oysters *Crasosstrea gigas* can boost immune system response and oocyte production. However, high levels of ARA favor final maturation and advanced stages of vitellogenesis but possibly at the expense of immune response. In addition, he also stated that arachidonic acid is essential for oysters and has been implicated in reproduction and immune response of different mollusk species, probably by modulating the levels of prostaglandins. Arachidonic acid is a precursor for synthesis of eicosanoids, mainly prostaglandins of the series II

(PGE_2). ARA is involved in the modulation of the immune system, with both pro-inflammatory and immunosuppressive properties. ARA supplementation in the diet increased heamocyte count, phagocytosis, and production of reactive oxygen species (ROS) by hemocytes in *C. gigas* and suggested that ARA directly or its eicosanoid metabolites affect oyster heamocyte functions. During reproduction, there is an inhibition of phagocytic activity and adhesive capacity of hemocytes in *C. gigas*. Immune response can also be a result of increased temperature, which in turn can modulate oocyte maturation, or increase prostaglandin levels. Either way, an increase in PGE_2 has been associated with gonad development and oocyte release during spawning in mollusks. Palacios et al. (2005) reported that accumulation of lipids in female gonads during gametogenesis is well documented for several species of pectinids. There are two postulated mechanisms by which pectinids accumulate lipids in the female gonads during maturation: (1) transfer of lipids from the digestive gland to the gonads, and (2) lipogenesis from carbohydrates stored in the muscle, which results in a decrease of muscle carbohydrates during gametogenesis. Lipogenesis or synthesis of saturated fatty acids can occur directly in the gonads or in other tissues.

However, mollusks have a limited capacity for elongation and desaturation of long-chain polyunsaturated fatty acids. Thus, PUFA for gonad development are probably obtained directly from diet or indirectly after previous accumulation in the digestive gland or other tissues. The role of diet in comparison to previously stored components has been addressed for general biochemical composition, but there are few studies that analyze tissue-specific fatty acid variations in relation to gametogenesis in pectinids, and none for giant lion's-paw scallop. Furuita et al. (2003) found that a supplement of ARA at 0.6 g/100 g diet improved the reproductive performance in broodstock of Japanese flounder *Paralichthys olivaceus*, but a higher

level of ARA (1.2 g/100 g diet) negatively affected both egg and larval quality due to a potential inhibitory effect on EPA bioconversion. They also concluded that addition of ARA to broodstock diets improved the egg quality of Japanese flounder. However, excess ARA may have negative effects on reproduction of Japanese flounder. Recent studies suggest the importance of the ratio of EPA to DHA as well as the ratio of n-3 series to n-6 series HUFA in larval and broodstock diets. Efforts should be directed toward establishing the optimum ratio of DHA/EPA/ARA in diets. More research effort is needed to establish formulated diets appropriate for broodstock of Japanese flounder. Furuita et al. (2007) also suggested that both n-3 and n-6 fatty acids are necessary for reproduction as well as growth of eel broodstock *A. japonica and,* and a higher ratio of n-6 to n-3 fatty acids negatively affected embryogenesis. In addition, both n-3 and n-6 PUFA are necessary as supplements for eel broodstock diets. A combination of plant and fish oils (i.e., corn oil and pollack oil) is better than plant oil or fish oil alone for the diets, from the point of view of egg quality and fatty acid composition of broodstock and eggs. Furuita et al. (2007) also suggested that both n-3 and n-6 fatty acids are necessary for reproduction as well as growth of Japanese eel broodstock *A. japonica,* and a higher ratio of n-6 to n-3 fatty acids negatively affected embryogenesis. In addition, Bell and Sargent (2003) stated on the effect of ARA on reproduction; egg, sperm and larval quality of marine fish. The importance of ARA in reproduction was first identified in European sea bass broodstock fed diets containing either northern hemisphere fish oil or a local trash fish, bogue (*Boops boops*). The trash fish diet contained around eightfold more ARA than the fish oil diet and the EPA/ARA ratios were 1.5 and 15 for the trash fish and fish oil diets, respectively. Dietary ARA was found to be highly concentrated in the eggs and sperm of sea bass broodstock fed trash fish and in wild-caught broodstock. Subsequently, pelleted dry

feeds were prepared with increased levels (twofold) of ARA which were successful in improving egg and larval quality in cultured European sea bass. Fish fed the ARA supplemented feed showed significantly increased concentrations of plasma eicosanoids, compared to broodstock fed northern hemisphere fish oils. While optimum dietary ARA concentration and ARA/EPA ratios are likely to be species-dependent, and influenced by the geography and ecosystem the species inhabits, there is evidence that a high ARA/EPA ratio in eggs may be ubiquitous. Analyses of eggs and larvae from eight species of northern hemisphere marine fish showed that ARA levels were several fold higher than in normal maternal tissue lipids. This suggests that ARA may be specifically concentrated in fish eggs, compared to tissues, and confirms a high biological activity for this HUFA in reproductive processes. In more recent studies, significant improvements in egg and larval quality have been seen in Atlantic halibut broodstock fed diets containing supplemental ARA. Halibut fed diets containing 2% of total fatty acids as ARA showed significant improvements in fertilization and hatching rates compared to those containing 0.5% or 1.0% ARA.

Furthermore, this study also indicated that high level of dietary 20:4n-6 and ARA/DHA ratio (0.86:1) had a positive effect on spawning performance. *B. areolata* broodstock with high level of 20:4n-6 improved spawning performance but not for egg and larval quality and fatty acid composition of eggs. Several studies suggested that there exist a delicate balance between n-3 and n-6 fatty acid families in penaeid shrimp tissues during ovarian maturation, and believed that maturation diets should contain high n-3/n-6 ratios (Lytle et al., 1990). As compared to the study of Wouters et al. (2001) and (Cahu et al, 1994), the n-3 to n-6 ratios in the mature ovaries of *Penaeus semisulcatus* and *Litopenaeus vannamei* spawners were approximately 2 to 1, respectively. Previous research on pond-reared *P. monodon* broodstock showed that

diets with the highest levels of 20:4n-6, 20:5n-3 and 22:6n-3 and with the highest n-3 : n-6 ratios resulted in the highest fertilization, hatching rates and spawning frequency (Millamena, 1989). Pickova, Brannas and Anderson (2007) also reported feed based on marine raw product did not fulfill the requirements for essential fatty acids for freshwater char *Salvelinus alpines* and suggested that ARA is supplemented to the broodstock diet and that at least linoleic acid (18:2n-6) is included in the on-growth diet to lower the n-3 / n-6 fatty acid ratio. Wang et al. (2010) reported that EPA level was slightly higher in the dry diet than in forage fish. Thus, there was a difference between the two diets in terms of EPA/ARA ratio, It is known that EPA and ARA compete for the same enzymatic system for production of three-series and two-series prostaglandins respectively. These prostaglandins do not share the same physiological functionalities. It was shown *in vitro* that prostaglandin E_2 (PGE_2) (derived from ARA) induces oocyte maturation in sea bass while PGE_3 (derived from EPA) does not. Therefore, an unbalanced EPA/ARA ratio in the diet in flavor of EPA could lead to a reduced biosynthesis of two-series prostaglandins leading to a lesser ability to undergo final maturation and spawning in female and a lowered sperm production and quality in male. Thus, the high level of EPA in the diet may partly explain a low spawning performance in this study. However, many other nutritional factors might also be involved. ARA did not enhance tolerance to low salinity stress and starvation test together with increasing the levels of dietary ARA in *B. areolata* broodstock. All dietary trials had no significantly differences in durations at 100% mortality of starvation stress test and low salinity stress test with ranging from 5.9 – 6.2 days and 5.5 – 6.0 days. As compared to the study of Zheng et al, 2005 impact of starvation on larvae of Ivory shell, *Babylonia formosae habei,* was percent metamorphosis 53.75% for the nonstarved, but all larvae died before 10 days. Henrotte et al. (2010) indicated

that a DHA/EPA/ARA ratio of 3/2/2 seemed to be effective for obtaining eggs and larvae of good quality in Eurasian perch (*Perca fluviatilis*). He also stated that low levels of ARA and DHA in the diet may reduce spawning quality. DHA plays an important role in the embryonic development as well as in the larval stage. Furthermore, the ratio EPA/ARA determines the equality of reproduction to a huge extent and the development of diets for breeders must be more species-specific. Rezek et al. (2009) also indicated that black sea bass (*Centropristis striata*) larvae fed prey containing 10% DHA with increasing ARA within the range of 0-6% showed improve growth and survival from first feeding through metamorphic stages.

Conclusion

The results suggest that addition of ARA to broodstock diet improved spawning quality in spotted babylon (*B. areolata*) but not for egg and larval quality. However, more attention should be paid to the DHA/EPA/ARA ratio in broodstock diet for improving egg and larval quality. More research effort is needed to establish formulated diets appropriate for broodstock of this species.

CHAPTER V

SUMMARY

1. This first attempt to condition *B. areolata* broodstock using formulated diets under hatchery conditions provided good results for replacement of the traditional use of trash fish to the formulated diets as well as providing the appropriate guidelines for development of a fully formulated broodstock diet for commercial applications in the future. Furthermore, the formulated diets had several advantages for broodstock conditioning in hatchery than those of the trash fish commonly used in the commercial hatcheries such as a reliable supply, low competition in supply, minimal preparation time, known nutrient contents, constant quality of diets and nutritional values, ease for storage with low expenses, low cost of transportation, need low volume of car or truck, opportunity to orally administer drugs such as hormones or supplemented vitamins, low pathogen infection from diets

2. Females fed on diet 5%TO showed the good results in spawning quality (Total number of spawning, monthly frequency of spawning and total egg capsules production) but not for egg and larval quality.

3. Females fed on diets 5%TO showed the best results of ARA, EPA, DHA, n-3 HUFA, n-6 HUFA and n-3 PUFA in egg capsules than those of females fed trash fish as well as other experimental diets.

4. More works should be concentrated on deep in details for the particular components and ratios of fatty acids on better egg and larval quality in this targeted species.

5. Supplement of arachidonic acid (ARA) at 1.6% providing total ARA, ARA/ EPA ratio and DHA/ EPA ratio of 387 mg/100g diet, 0.86 and 3.9 improved spawning quality of broodstock but not for egg and larval quality. Excess ARA may have negative effects on egg and larval quality because recent studies report the importance of the ratio of EPA / DHA, ARA/ EPA and ratios of n-3 series to n-6 series HUFA in larval and broodstock diets.

6. Efforts should be direct toward establishing the optimum ratio of DHA / EPA/ARA in diets. Furthermore research effort is needed to establish formulated diets appropriate for broodstock of this species

7. Furthermore, variability in egg and larval quality may depend on many factors such as other essential nutrients, environmental factors and stresses, age of broodstock, uncontrolled genetic, etc.

REFERENCES

Association of Official Analytical Chemists. 1990. Official methods of analysis. 15th edition. Association of Official Analytical Chemists. Arlington. VA, 931 pp.

Ballestrazzi, R., Rainis, S., Tulli, F. and Bracelli, A. 2003. The effect of dietary coconut oil on reproductive traits and egg fatty acid composition in rainbow trout (*Oncorhynchus mykiss*). Aquaculture International 11: 289-299.

Bell, J. G., Farndale, B. M., Bruce, M. P., Navas, J. M. and Carrillo, M. 1997. Effects of broodstock dietary lipid on fatty acid compositions of eggs from sea bass (*Dicentrarchus labrax*). Aquaculture 149: 107-119.

Bell, J. G. and Sargent, J. R. 2003. Arachidonic acid in aquaculture feeds: current status and future opportunities. Aquaculture 218: 491-499.

Berntsson, K. M., Jonsson, P. R., Wangberg, S. A. and Carlsson, A. S. 1997. Effects of broodstock diets on fatty acid composition, survival and growth rates in larvae of the European flat oyster, *Ostrea edulis*. Aquaculture 154: 139-153.

Bromage, N. R. and Roberts, R. J. 1995. Broodstock management and egg and larval quality. Oxford: Blackwell Science, 424 pp.

Cahu, C., Guillaume, J. C., Stephan, G. and Chim, L. 1994. Influence of phospholipid and highly unsaturated fatty acids on spawning rate and egg and tissue composition in *Penaeus vannamei* fed semi-purified diets. Aquaculture 126: 159-170.

Chaitanawisuti, N. and Kritsanapuntu, A. 1997. Laboratory spawning and juvenile rearing of the marine gastropod: Spotted babylon, *Babylonia areolata* Link, 1807 (Neogastropoda : Buccinidae) in Thailand. Journal of Shellfish Research 16: 31-37.

Chaitanawisuti, N., Kritsanapuntu, A. and Natsukari, Y. 2002. Economic analysis of a pilot commercial production for spotted babylon, *Babylonia areolata* Link 1807, marketable sizes using a flow-through culture system in Thailand. Aquaculture Research. 33: 1-8.

Christie, W.W. 1982. Lipid analysis. Oxford: Pergamone press, 207 pp.

Christie, W.W. 1989. Gas chromatography and lipids: a practical guild. UK: The Oily Press, 315 pp.

Delaunay, F., Marty Y., Moal J., and Samain J. F. 1992. Growth and lipid class composition of *Pecten maximus* larvae grown under hatchery conditions. Journal of Experimental Marine Biology and Ecology 163: 209-219.

Djunaidah, I. S., Wille, M., Kontara, E. K. and Sorgeloos, P. 2003. Reproductive performance and offspring quality in mud crab (*Scylla paramamosain*) broodstock fed different diets. Aquaculture International 11: 3-15.

Duame, S. and Ryan, S. 2004. Fatty acid composition of eggs derived from conditioned and wild caught greenlip abalone broodstock (*Haliotis laevigata*). Journal Shellfish Research 23: 967-974.

Dunstan, G.A., Baillie, H.J., Barrett, S. M. and Volkman, J. K. 1996. Effect of diet on the lipid composition of wild and cultured abalone. Aquaculture 140: 115-127

Emata, A. C., Ogata, H. Y., Garibay, E. S. and Furuita, H. 2003. Advanced broodstock diets for the mangrove red snapper and a potential importance of arachinodic acid in eggs and fry. Fish Physiology and Biochemistry 28: 489-491.

Furuita, H., Yamamoto, T., Shima, T., Suzuki, N. and Takeuchi, T. 2002. Effect of high levels of n-3 HUFA in broodstock diet on egg quality and egg fatty acid composition of Japanese flounder *Paralichthys olivaceus*. Aquaculture 210: 323-333.

Furuita, H., Yamamoto, T., Shima, T., Suzuki, N. and Takeuchi, T. 2003. Effect of arachidonic acid levels in broodstock diet on larval and egg quality of Japanese flounder *Paralichthys olivaceus*. Aquaculture 220: 725-735.

Furuita, H., Unuma, T., Nomura, K., Tanaka, H., Okuzawa, K., Sugita, T. and Yamamoto, T. 2006. Lipid and fatty acid composition of eggs producing larvae with high survival rate in the Japanese eel *Anguilla japonica*. Fish Biol. 69: 1178-1189.

Furuita, H., Hori, K., Suzuki, T., Sugita, T., and Yamamoto, T. 2007. Effects of n-3 and n-6 fatty acids in broodstock diet on reproduction and fatty acid composition of broodstock and eggs in the Japanese eel *Anguilla japonica*. Aquaculture. 267: 55-61.

Gallager, S. M. and Mann, R. 1986. Growth and survival of larvae of *Mercenaria mercenaria* (L.) and *Crassostrea virginica* (Gmelin) relative to broodstock conditioning and lipid content of eggs. Aquaculture 56: 105-121.

Gittenberger, E. and Goud, J. 2003. The genus Babylonia revisited (Mollusca: Gastropoda: Buccinidae). Zoologische Verhandelingen 345: 151-162.

Habe, T. 1965. Notes on the ivory shell genus Babylonia Schluter (Mollusca). Bull. Nat. Sci. Mus. 8: 115-125.

Henrotte, E, Mandiki, R.S.N, Prudencio, A, Vandecan, M, Melard, C, and Kestemont, P. 2010. Egg and larval quality, and egg fatty acid composition of Eurasian perch breeders (*Perca fluviatilis*) fed different dietary DHA/EPA/ARA ratios. Aquaculture Research doi: 10.1111/j.1365-2109.2009.02455.x.

Hurtado, M. A., Reza, M., Ibarra, A. M., Wile, M. Sorgeloose, P., Soudant, P. and Palacios, E. 2009. Arachidonic acid effect on reproduction, immunology and prostaglandin E levels in *Crassostrea corteziensis* (Hertlein 1951). Aquaculture 294: 300-305.

Izquierdo, M. S., Ferna´ndez-Palacios, H. and Tacon, A. G. J. 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197: 25–42.

Larvens, P., Jaunet, E. L. H., Brunel, A., Dhert, P. and Sorgeloos, P. 1999. Effect of dietary essential fatty acids and vitamins on egg quality in turbot broodstocks. Aquaculture International 7: 225-240.

Li, Y. Y., Chen, W. Z., Sun, Z. W., Chen, J. H., and Wu, K. G. 2005. Effects of n-3 HUFA content in broodstock diet on spawning performance and fatty acid composition of eggs and larvae in *Plectorhynchus cinctus*. Aquaculture 245: 263-272.

Ling, S., Hashim, R., Kolkovski, S. and Chien, A.C.S. 2006. Effect of varying dietary lipid and protein levels on growth and reproductive performance of female swordtails *Xiphophorus helleri* (Poeciliidae). Aquaculture 37: 1267-1275.

Lytle, J. S., Lytle, T. S. and Ogle, J.T. 1990. Polyunsaturated fatty acid profiles as a comparative tool in assessing maturation diets of *Penaeus vannamei*. Aquaculture 89: 287-299.

Marasigan, E. T. and Laureta, L.V. 2001. Broodstock maintenance and early gonadal maturation of *Pholas orientalis* (Bivalve:Pholadidae). Journal of Shellfish Research 20: 1095-1099.

Mazorra, C., Bruce, M., Bell, J.G., Davie, A., Alorend, E., Jordan, N., Rees, J., Papanikos, E., Porter, M. and Bromage, N. 2003. Dietary lipid enhancement of broodstock reproductive performance and egg and larval quality in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 227: 21-33.

Millamena, O. M. 1989. Effect of fatty acid composition of broodstock diet on tissue fatty acid patterns and egg fertilisation and hatching in pond-reared *Penaeus monodon*. Asian Fisheries Science 2: 127-134.

Palacios, E., Racotta, I. S., Kraffe, E. K., Marty, Y., Moal, J. and Samain, J. F. 2005. Lipid composition of the giant lion's – paw scallop (*Nodipecten subnodosus*) in relation to gametogenesis I. Fatty acid. Aquaculture 250: 270-282.

Pickova, J., Brannas, E. and Anderson, T. 2007. Importance of fatty acids in broodstock diets with emphasis on Arctic char (*Salvelinus alpines*) eggs. Aquaculture International 15: 305-311.

Prescott, S. M., The COXes of Daio: From mechanistic model to experimental therapeutics [online], January 2010. www.pnas.org/ content /99/14/9084.full.pdf+html.

Rezek, T.C., Watanabe, W.O., Harel, M. and Seaton, P. 2009. Effects of dietary docosahexanenoic acid (22:6n-3) and arachidonic acid (20:4n-6) on the growth, survival, stress resistance and fatty acid compositin in black sea bass

Centropristis striata (Linnaeus 1758) larvae. Aquaculture Research Doi:10.1111/j.1365-2109.2009.02418.x.

Salze, G., Tocher, D. R., Roy, J. R. and Robertson, D. A. 2005. Egg quality determinants in cod (*Gadus morhua* L.): egg performance and lipids in eggs from farmed and wild broodstock. Aquaculture Research: 36: 1488-1499

Sanghvi, K. H., An overview of Antioxidants in healthcare - Uses and Estimation [online], January 2010. www.pharmainfo.net/pharma-student- magazine/ overview-antioxidants-healthcare-uses-and-estimation.

Soudant, P., Marty, Y., Moal, J., Robert, C., Quere, J. R., LeCoz, J. R. and Samain, J. F. 1996. Effect of food fatty acid and sterol quality on *Pecten maximus* gonad composition and reproductive process. Aquaculture 143: 361-378.

Teruel, M. N. B., Millamena, O. M. and Fermin, A. C. 2001. Reproductive performance of hatchery-bred donkey's ear abalone, *Haliotis asinine* Linne, fed natural and artificial diets. Aquaculture Research 32: 249-254.

Utting, S. D. and Millican, P. E. 1997. Techniques for the hatchery condition of bivalve broodstocks and the subsequent effect on egg quality and larval viability. Aquaculture 155: 45-54.

Utting, S. D. and Millican, P. E. 1998. The role of diet in hatchery conditioning of *Pecten maximus* : a review. Aquaculture 165: 167-178.

Wang, N., Mandiki, S.N.M., Henrotte, E., Bouyahia, A.G., Mairesse, G., Rougeot, C., Melard, C. and Kestemont, P. 2009. Effect of partial or total replacement of forage fish by a dry diet on the quality or reproduction in pikeperch, *Sander lucioperca*. Aquaculture Research 40: 376-383.

Wilson, J. A., Chaparro, O. R. and Thompson, R. J. 1986. The importance of broodstock nutrition on the viability of larvae and spat in the Chilean oyster *Ostrea chilensis*. Aquaculture 139: 63-75.

Wouters, R., Molina, C., Lavens, P. and Calderon, J. 2001. Lipid composition and vitamin content of wild female *Litopenaeus vannamei* in different stages of sexual maturation. Aquaculture 198: 307-323.

Xu, X. L., Ji, W. J, Castell, J. D. and O'Dor, R. K. 1992. Influence of dietary lipid sources on fecundity egg hatchability and fatty acid composition of Chinese prawn (*Penaeus chinensis*) broodstock. Aquaculture 119: 359-370.

Xu, X. L., Ji, W. J, Castell, J. D. and O'Dor, R. K. 1994. Essential fatty acid requirement of the Chinese prawn (*Penaeus chinensis*) broodstock. Aquaculture 127: 29-40.

Zheng, H., K., C., Zhou, S. and Li, F. 2005. Effects of starvation on larval growth, survival and metamorphosis of Ivory shell *Babylonia formosae habei* Altena et al., 1981 (Neogastropoda: Buccinidae). Aquaculture 243: 357-366.

APPENDICES

APPENDIX A

FATTY ACIDS DETERMINATION

Determination of fatty acids in feeds and shell tissues by GC (AOAC, 1990)

1. Principle

Fat and fatty acids are extracted from food by hydrolytic methods. Pyrogallic acid is added to minimize oxidative degration of fatty acids during analysis. Triglyceride, triundecanoin (C11:0), is added as internal standard. Fat is extracted into ether, then methylated to fatty acid methyl esters (FAMEs) using BF_3 in methanol. FAMEs arequantitatively measured by capillary gas chromatography (GC) against $C_{11:0}$ internal standard. Total fat is calculated as sum of individual fatty acids express as triglyceride equivalents. Saturated and monounsaturated fats are calculaterd as sum of respective fatty acids. Monounsaturated fat includes only cis form.

2. Apparatus

1). Gas chromatography (GC): Equipped with hydrogen flame ionization detector, capillary column, split mode injector, oven temperature programming sufficient to implement a hold-ramp-hold sequence. Operating condition: (^{0}C): injector, 225; detector, 285; initial temp, 100 (hold 4 min); ramp, 3^0C/min; final temp 240; hold 15 min; carrier gas, helium; flow rate, 0.75 mL/min; linear velocity, 18 cm/s; split ratio, 200: 1

2). Capillary column : Separating the FAME pair of adjacent peaks of C18:3 and C20:1 and the FAME trio of adjacent peaks of C21:1 and C20:3, and C20:4 with a resolution of 1.0 or greater. SP2560 100 m x 0.25 mm with 0.20 µm flim is suitable.

3). Mojonnier flasks

4). Stoppers

5). Mojonnier centrifuge basket

6). Hengar micro boiling granules

7). Baskets

8). Shaker water bath

9). Steam bath

10). Water bath

11). Wrist action shaker

11). Mojonnier motor driven centrifuge

12). Vortex mixwer

13). Gas dispersion tubes

14). Phenolic closed top caps

15). Teflon/ silicone septa

3. Reagents

1). Pyrogllic

2). Hydrochloric acid:

3). Ammonium hydroxide

4).Diethyl ether

5). Petroleum ether

6). Toluene

7). Chloroform

8). Sodium sulfate

9). Boron trifluoride reagent

10). Diethyl ether

11). Triglyceride internal standard solution

12). Fatty acid methyl esters (FAMEs) standard solution.

- Mixed FAMEs standard solution: Reference mixture containing series of FAMEs, includeing C18:1 cis and trans (available as GLC-85 from Nu Chek Prep, Elysian, MN56028, USA or equivalent)

-C11:0 FAMEs standard solution: C11:0-Undecanoic methyl ester in hexane.

-Individual FAMEs standard solution: Standard solution of each of following FAMEs.

4. Extraction of Fat

5. Methylation

Dissolved extracted fat residue in 2-3 mL chloroform and 2-3 mL diethyl ether. Transfer mixture to 3 dram grass vial and then evaporate to dryness in 40^{o}C water bath under nitrogen stream. Add 2.0 mL 7% BF_3 reagent and 1.0 mL toluene. Seal vial with screwcap topcontaining Teflon/silicone septum. Heat vial in oven 45 min at 100^{o}C. Gently shake vial ca every 10 min.

6. GLC Determination

Relative retention time and response factors of individual FAMEs can be obtained by GC analysis of individual FAME standard solutions and mixed FAME standard solution. Inject ca 2 µL each of individual FAME standard solution and 2 µL of mixed FAME standard solution. Use mixed FAME standard solution to optimize chromatographic response before injecting any test solution. After all chromatographic conditions have been optimized, inject test solutions from methylation

7. Calculations

Total fat is the sum of fatty acids from all sources, expressed as triglycerides. Expressing measured fatty acids as triglycerides requires mathematical equivalent of

condensing each fatty acid with glycerol. For every 3 fatty acid molecules, 1 glyceral is required. Essential, 2 methylene groups and 1 methin group are added to every 3 fatty acids.

Calculate retention times for each FAME in individual FAMEs standard solutions, by subtracting retention of $C_{11:0}$ peak from retention time of fatty acid peak. Use these retention time to identify FAMEs in mixed FAMEs standard solution. Use additional FAME solutions when necessary for complete FAME identity verification.

1). Calculate response factor (Ri) for each fatty acid i as follows:

$$Ri = \frac{Ps_i}{Ps_{c11:0}} \times \frac{W_{c11:0}}{W_i}$$

where Ps_i = peak area of individual fatty acid in mixed FAMEs standard solution; $Ps_{c11:0}$ = peak area of $C_{11:0}$ fatty acid in mixed FAMEs standard solution; $W_{c11:0}$ = weight of internal standard in mixed FAMEs standard solution; $_{Wi}$ = weight of individual FAME in mixed FAMEs standard solution.

2). Calculate amount of individual in test portion as follows:

$$W_{FAMEi} = \frac{Pt_i \times Wt_{c11:0} \times 1.0067}{Pt_{c11:0} \times R_i}$$

$$W_{TGi} = W_{FAMEi} \times f_{TGi}$$

where $Pt_{i\ =}$ peak area of fatty acid *i* in test portion; $Wt_{c11:0}$ = weight of $C_{11:0}$ internal standard added to test portion; $Pt_{c11:0}$ = peak area of $C_{11:0}$ internal standard in test portion; and f_{TGi} = conversion factor for FAMEs

3). Calculate amount of total fat in test portion as follows:

$$\text{Total fat, \%} = (\Sigma WD_{TG}/W_{\text{test portion}})$$

where $W_{\text{test portion}}$ = weight of test portion

4). Calculate weight of each fatty acid (W_i) as follows:

$$W_i = W_{FAMEi} \times f_{FAi}$$

where f_{Fai} = conversion factor for conversion of FAMEs

5). Calculate percent of saturated fat in test portion

$$\text{Saturated fat, \%} = (\Sigma \text{saturated } W_i / W_{\text{test portion}}) \times 100\%$$

6). Calculate amount of monounsaturated fat in test sample

$$\text{Monounsaturated fat, \%} = (\Sigma \text{monounsaturated } W_i / W_{\text{test portion}}) \times 100\%$$

$$\text{Polyunsaturated fat, \%} = (\Sigma \text{polynsaturated } W_i / W_{\text{test portion}}) \times 100\%$$

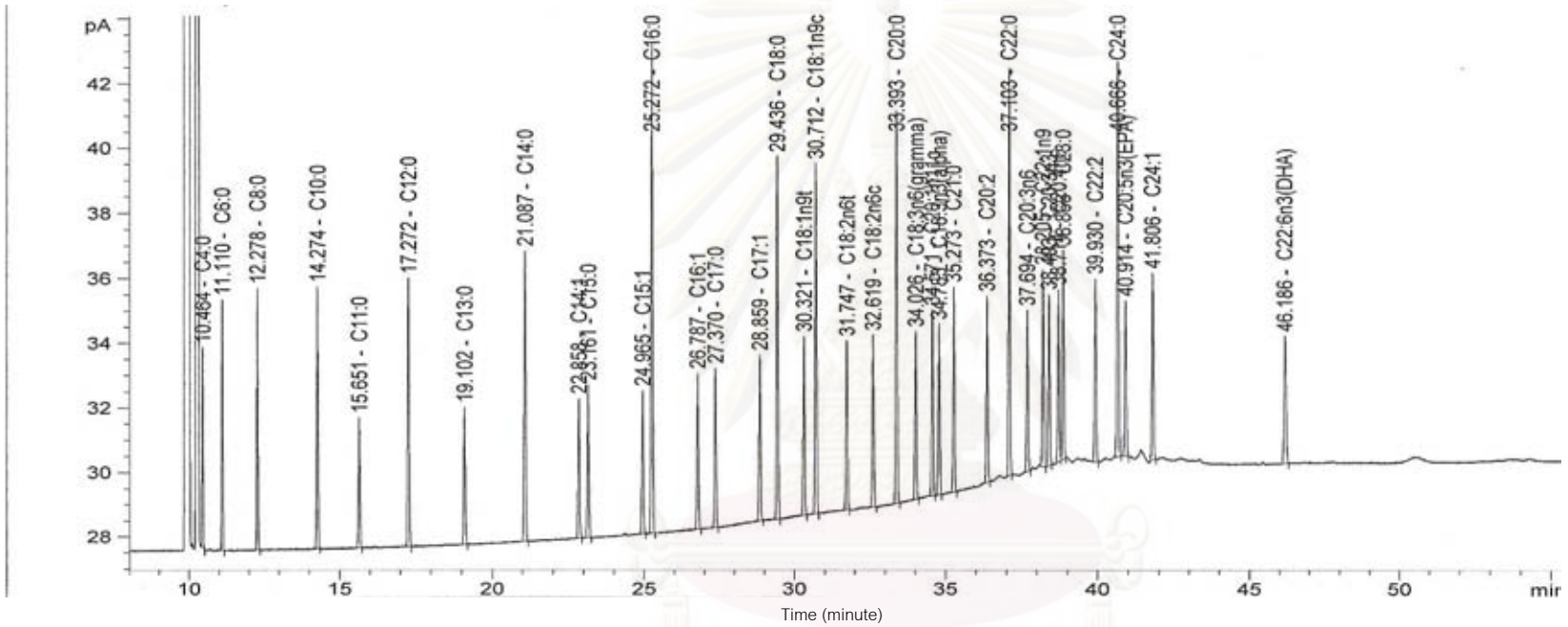


Figure_. Chromatogram of standard fatty acid

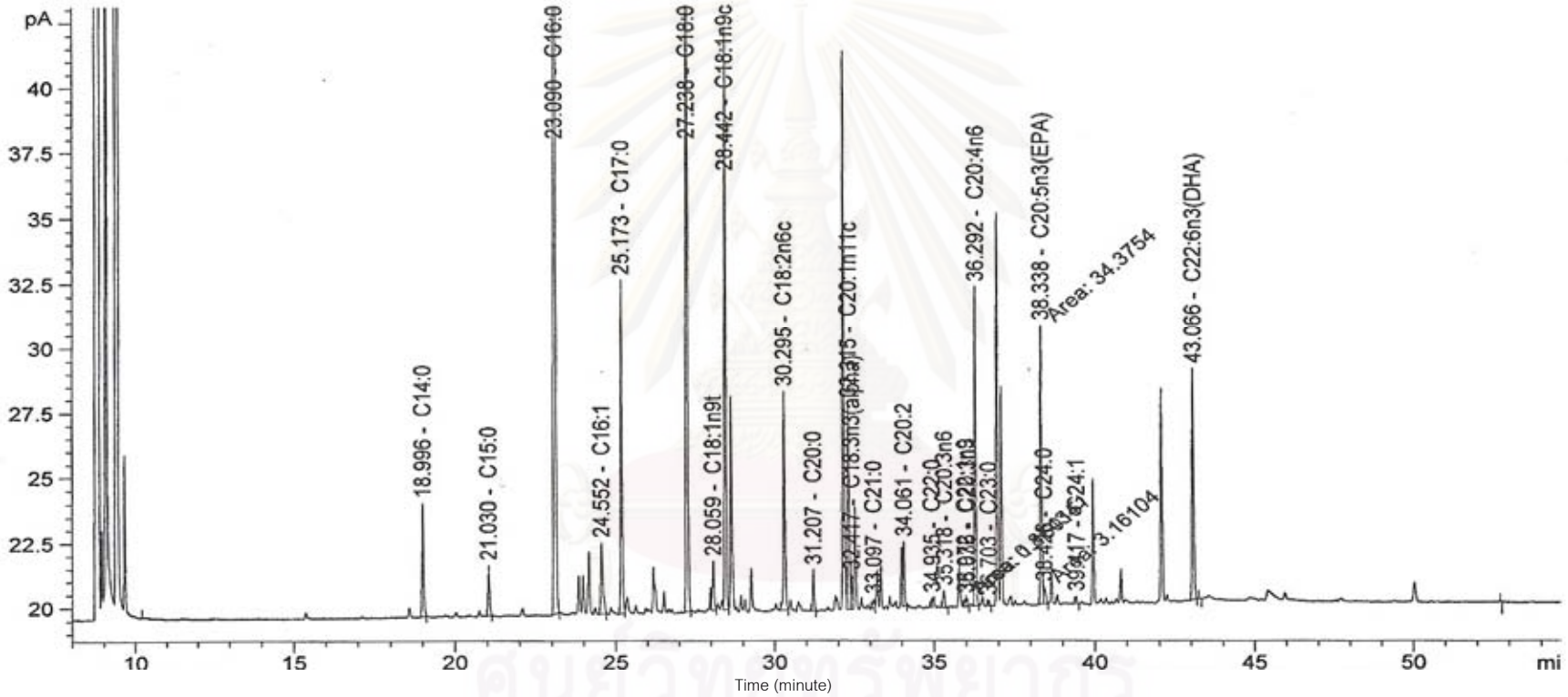


Figure_. Chromatogram of fatty acid in egg capsules obtained from spotted babylon females fed with ARA supplement diet

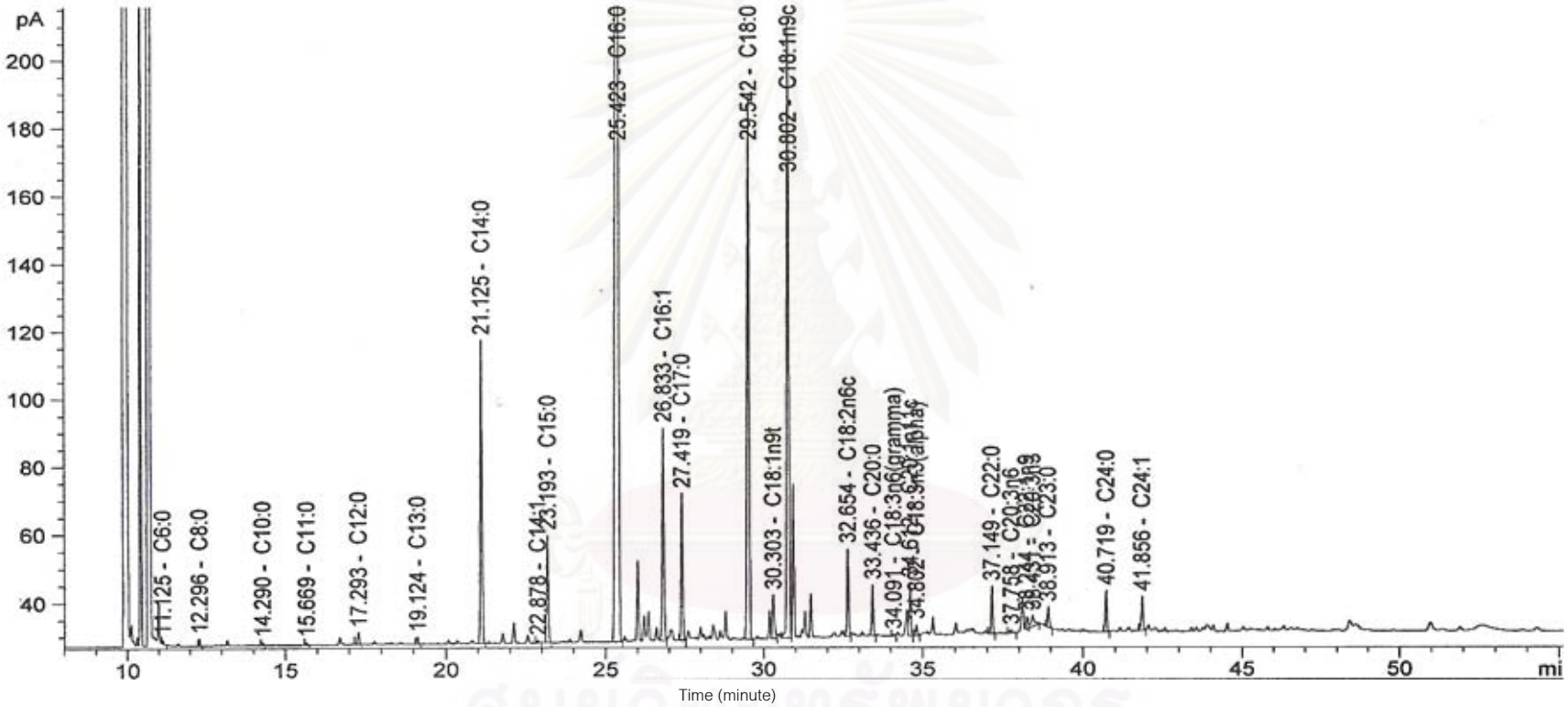


Figure_. Chromatogram of fatty acid in broodstock diet supplemented with ARA for spotted babylon

APPENDIX B

PROXIMATE ANALYSIS DETERMINATION

Crude protein

1. Reagent

(a) Catalyst tables. – Containing 3.5 g K_2SO_4 and 0.175 g HgO (Kjeltabs "MT" available from Tecator, Inc., 2875C Towerview Rd, Herndon, VA 22071, USA, or equivalent).

(b) Boric acid solution.-4%. Dissolve 4 g H_3BO_3 in H_2O containing 0.7 mL 0.1% alcoholic solution of methyl red and 1.0 mL 0.1% alcoholic solution of bromocresol green, and dilute to 100 mL with H_20

(c) Sodium hydroxide-sodium thiosulfate solution. – Dissolve 2000 g NaOH and 125 g $Na_2S_2O_3$ in H_2O and dilute to 5 L (ca 50 mL in used per analysis).

(d) Hydrochloric acid standard solution. – 0.2M (936.15 [see A.a.06]

(e) Hydrogen peroxide. – 30-35%.

(f) Sulfuric acid. Concentrated.

2. Apparatus

(a) Digestion block and associated glassware. – Tecator DS-6 or DS-20 (Tecator), or equivalent.

(b) Distillation unit and associated glassware. – Kjeltec 1003 (Tecator), or equivalent.

3. Determination

Accurately weigh ca 2 d well-ground and mixed test sample on 7 cm N-free filter paper (e.g., Whatman 541), fold, and transfer to 250 mL digestion tube. Place tubes in fume hood and add 2 or 3 boiling chips, 2 catalyst tablets, 15 mL H_2SO_4 , and slowly 3 mL 30-35% H_2O_2 . Let reaction subside and place tubes in block digestor preheated at 410 ^{0}C. (Digestor must be place in perchloric acid fume hood or be equipped with exhaust system. Rapid addition of 30-35% H_2O_2 may cause the reaction to become violent.) Digest at 410 ^{0}C until mixture is clear, ca 45 min. Remove tubes and let cool ca 10 min. Do not let precipitate form; if precipitate forms, reheat. Carefully add 50-75 mL H_20.

Place NaOH - $Na_2S_2O_3$ solution in alkali tank of steam distillation unit. Make sure that 50-75 mL is dispensed from unit before conducting distillation. Attach digestion tube containing diluted digest to distillation unit. Place 250 mL receiving flask containing 25 H_3BO_3 solution with mixed indicator on receiving platform, with tube from condenser extending below surface ob absorbing solution. Steam distil until 100-125 mL collects (absorbing solution turns green from liberated NH_3). Remove digestion tube and receiving flask from unit.

Titrate absorbing solution with 0.2M HCl to neutral gray end point and record volume acid required to 0.01 mL. Titrate reagent blank similarly.

N, % = (V_A-V_B) x 1.4007 x M/g test portion

Protein, % = (V_A-V_B) x 1.4007 x M x 6.25/ g test portion

where V_A and V_B = volume standard acid required for test portion and blank, respectively; 1.4007 = milliequivalent weight N x 100(%); M = molarlity of standardized acid; and 6.25 = protein factor for meat products (16% N).

Crude fat

1. Preparation of Test Sample

Prepare test sample according to type of pack and keep ground material in sealed jar. If jar has been chilled, let contents come to room temperature and shake jar. Open jar and stir contents with spatula, thoroughly scraping sides and lid so as to incorporate any separated liquid or fat.

2. Determination

Weight 8 g well-mixed test portion into 50 mL beaker and add 2 mL HCL. Using stirring rod with extra large flat end, break up coagulated lumps until mixture is homogeneous. Add additional 6 mL HCL, mix, cover with watch glass, and heat on steam bath 90 min, stirring occasionally with rod. Cool solution and transfer to Mojonnier fat-extraction flask. Rinse beaker and rod with 7mL alcohol, add to extraction flask, and mix. Rinse beaker and rod with 25 mL ether, added in 3 portion; add rinsings to extraction flask, stopper with cork or stopper of synthetic rubber unaffected by usual fat solvents, and shake vigorously 1 min. Add 25 mL petroleum ether to extraction flask and repeat vigorous shaking. Centrifuge Mojonnier flask 20 min at ca 600 rpm

Drying to constant weight take ca 40 min. Long heating period may increase weight of fat. If centrifuge is not available, extraction can generally be made by letting Mojonnier flask stand until upper liquid is practically clear, thenswirling flask and again letting stand until clear. If troublesome emulsion forms, let stand, pour off as much of ether-fat solution as possible, add 1-2 mL alcohol to Mojonnier flask, swirl and again let mixture separate.

Ash

- Heat test portion of appropriate weight for product being examined (usually 5-10 g) in 50-100 mL platinum dish at 100^{o}C until water is expelled.

- Add a few drops of pure olive oil and heat slowly over flame or under infrared lamp until swelling stops.

- Place dish in furnace at ca 525 ^{o}C and leave until white ash is obtained

- Cool, moisten ash with water, dry on steam bath and then on hot plate

- Re-ash at 525 ^{o}C to constant weight.

Ash was determined by muffle furnace

Moisture

Air Drying

- With lids removed, spread test portion out over base of dish and dry test portion containing ca 2 dry material 16-18 h at 100-102^{o}C in air oven . Use covered A1 dish ≥50 mm diameter and ≤40 mm deep. Cool in desiccator and weight. Report loss in weight as moisture, g.

BIOGRAPHY

Mr. Sarawut Sangsawangchote was born on January 12, 1973 in Angthong Province. He graduated with a Bachelor degree and Master degree in Aquatic Science from Department of Aquatic Science, Faculty of Science, Burapha University, Chonburi Province. In 2005, he started the Ph.D. program of Marine Science at the Department of Marine Science, Faculty of Science, Chulalongkorn University.

Research Publications:

Sangsawangchote, S., Chaitanawisuti, N. and Piyatiratitivorakul, S. 2010. Reproduction performance, egg and larval quality and egg fatty acid composition of hatchery-reared spotted babylon (*Babylonia areolata*) broodstock fed natural and formulated diets under hatchery conditions. Journal of Fisheries and Aquaculture 2 (1): 049-057.