

DEVELOPMENT OF AQUACULTURE TECHNOLOGY FOR THE HAWAIIAN OPIHI  
*CELLANA SPP.*

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY  
OF HAWAI'I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR  
THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MOLECULAR BIOSCIENCES AND BIOENGINEERING

AUGUST 2014

By

Nhan Thai Hua

Dissertation Committee:

Harry Ako, Chairperson  
Clyde Tamaru  
Jon-Paul Bingham  
Cheng-Sheng Lee  
Theodore Radovich

Keywords: Opihi, Aquaculture, Formulated feed, Spawning season, final maturation

Student: Nhan Thai Hua  
Student ID #: 20846058  
Degree: PhD  
Field: Molecular Biosciences and Bioengineering  
Graduation date: August 2014

Title: Development of Aquaculture Technology for the Hawaiian Opihi *Cellana* spp.

We certify that we have read this dissertation and that, in our opinion, it is satisfactory in scope and quality as a dissertation for the degree of Doctor of Philosophy in Molecular Biosciences and Bioengineering.

Dissertation Committee:

Names

Signatures

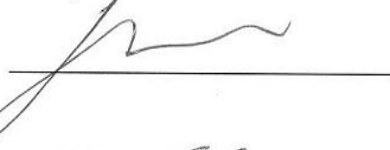
Harry Ako, Chairperson



Clyde Tamaru



Jon-Paul Bingham



Cheng-Sheng Lee



Theodore Radovich



## **DEDICATION**

This one first goes to my grandfather Hua Yen Nghi who just passed away forever. To my lovers: my father Hua Hoc Nghia and mom Truong Thi Phuong, to my brothers and sister who have always stay by my side no matter what, as well as my uncles, aunties and friend ST who have given your encouragement and support me during my study.

## ACKNOWLEDGMENTS

This dissertation would not have been possible without the help and encouragement of many people. First and foremost, I would like to express my greatest gratitude to my advisor, **Professor Dr. Harry Ako**, for his agreement to take me on as a Ph.D student and give me a two years assistantship, for his supervision, comments, advice and for scolding me to get me to do deep critical thinking. His efforts made this project run smoothly and I want to thank him for all the help he has given me. His supervision was invaluable.

I would also like to express my sincere thanks to the other members of my supervisory committee: Dr. Clyde Tamaru for his advice on breeding techniques, especially in chapter 6 and for his many useful seminars on practical aquaculture; Dr. Jon-Paul Bingham, for teaching me one of the most important courses, biochemistry, which is about the basic phenomenon of life, that I did not really appreciate until I became involved in real research topics, and also for other courses including MBBE 610, as well as his useful advice for my research; Dr. Cheng-Sheng Lee, for his valuable supervision of my research and for giving me a broad vision of aquaculture; and Dr. Theodore Radovich for his comments, guidance and hospitality. Thank you all for the opportunities you have given to me.

I deeply appreciate a special person who is actually not officially in my committee but who gave me so much by acting and teaching with all his heart, Dr. Dulal Borthakur, thank you for your teaching me several important courses, not only science but also the values of life. I sincerely thank you for all the help that you and Pritty have had given me.

I would like to thank the Center for Tropical and Subtropical Aquaculture, USA, grant #2009-210 and the Vietnam Educational Foundation and the East West Center for financial support in the research of this dissertation and other expenditures.

I am greatly indebted to my friends at the Hawai'i Institute for Marine Biology, Dr. Jon-Paul Bingham's laboratory, Dr. Samir Khanal's laboratory, Prof. Dr. Celia Smith's laboratory and to Charles Nelson for providing facilities and equipment as well as your time and your expertise.

I would like to thank Mr. Adam Baker for introducing me to Dr. Ako; Dr. Christopher Bird for initiating animal collection and teaching me how to collect opihi in the proper way to minimize damaging them; Dr. PingSun Leung for teaching me statistic; Prof. Dr. Addison

Lawrence (Texas A&M University) for providing sea urchin feed; Dr. Warren Dominy, Dr. Dong Fang and Dr. Ju at Oceanic Institute for amino acid and fatty acid analysis; Vernon Sato for useful discussions; Cecilia Viljoen (Big Island Abalone Co.) for useful suggestion; Dr. Audrey Asahi (Kawelo Marine Laboratory, University of Hawaii at Manoa) for kindly providing algae and Dr. David Shimizu at the Pathology Laboratory, Queen's Hospital, for histology analysis.

Great thanks to other friends for their help with collecting opihi, to Nelson Ka'ai and Jason S. Levy for providing opihi, to Chinh Tran, to Hung Vo and others who accompanied me while I was in danger while collecting samples for my research.

I would also thank all my friends at the Vietnamese Student Association in Hawaii, my Pops Herman Libarios and his family and all the MBBE professors and staff for all the help and support they have given to me during my studies.

I would like to thank my friend Jimmy Olson for his help with English editing on several sections of my dissertation and his friendship, for all the help and encouragement he gave me.

I especially want to thank Assoc. Prof. Dr. Tran Ngoc Hai, Prof. Dr. Nguyen Thanh Phuong and all of my colleagues at College of Aquaculture and Fisheries, Can Tho University for the opportunity and support you all have given to me.

To all the people I have known in Hale Manoa dormitory, EWC, and others during my study in Hawai'i. You guys really showed the greatest spirit of ALOHA.

To others, if I miss you in the list of the acknowledgments, please forgive me and I sincerely apologize.

Mahalo Nui Loa!!!

## ABSTRACT

The purpose of this work was to develop effective technologies for commercial aquaculture of the Hawaiian limpet *Cellana* spp., known as opihi in Hawai'i. In this dissertation, our aim was to complete the life cycle of the yellow foot opihi *Cellana sandwicensis*. The first objective was to learn how to collect and develop an appropriate handling technique to maintain wild opihi to be used in feeding trials and also for breeding stock. The first collection trial showed a 58 % mortality, presumably due to injuries to animals while removing them from the rock. There was additional mortality of 42 % during transfer from one tank to another because they clung tightly onto the tank's wall. We were eventually able to achieve a minimum collection mortality of 30 % by being careful, however, it may be overestimated due to varying collection conditions. A holding system using plastic liners and soft plastic container led to zero mortalities due to handling in the subsequent trials. The next objective was to develop a commercial feed available for opihi. The analysis of the stomach contents found that benthic diatoms were the preferred feed in the wild and experimental feed trials on benthic diatoms called "biofilm" grown in the laboratory found that the opihi fed well at 0.47% dry matter/body weight/day (DM/BW/day) and survived well on it. Biofilm was the key to feed consumption when it was incorporated into artificial feed. While biofilm worked well as a maintenance diet, it is a natural feed that needed to be cultured and was unreliable over the long term. We found that a commercial preparation of the algae *Porphyra* commonly known as Nori was as attractive as biofilm and was an effective replacement for biofilm as a feeding stimulant. A formulated diet of fishmeal, soybean meal and krill meal including *Porphyra* produced the best growth rates ( $0.44 \pm 0.37$  %/day) and feed intake ( $0.73 \pm 0.53$  % DM/BW/day) over 10 weeks. Addition of krill meal combined with *Porphyra* into the diet produced a higher feeding rate (about 5 times) and promoted a significantly higher growth rate than those diets without krill meal. Among the experimental dietary protein levels from 21 % to 50 % and carbohydrate levels ranging from 18 % to 37 % diets were tested. Weight gain and specific growth rates of opihi increased with increasing dietary protein from 21 % diet and reached to maximum at the 35 % diet, and significantly ( $P < 0.05$ ) decreased at the 50 %. The fastest growth rates of animals were obtained with 27 % ( $0.27$  % day<sup>-1</sup>) and 32 % ( $0.26$  % day<sup>-1</sup>) and were significantly higher than 18 % and 37 % carbohydrate diets. We conclude that about 35 % protein and 32 % carbohydrate levels

could be used for opihi and could be a model for aquatic herbivores. Monthly measurement of gonadosomatic index (GSI) and histology analysis of the gonad of the animals found that the peak spawning season for opihi occurs from November to December and seems to extend to January. The non-reproductive season is proposed to take place from late February to early September, followed by a final maturation phase which occurs in October. This finding provided an important exogenous key factor for developing an effective induction of opihi gonad maturation in the laboratory. Final maturation was successfully induced by adding ARA (arachidonic acid) into the diet at an ARA/EPA (ecosapentaenoic acid) ratio of 0.70. The GSI values of opihi reached to final maturation stage of 24.5 % and 23.7 % for 0.24 % ARA and 0.39 % ARA diets respectively, and were significantly higher than GSI of opihi (6.11 %) that were fed with the control diet without supplementation of ARA after 75 days. Poor reproductive performance was obtained when the animals were exposed to the wrong natural photoperiod. Another trial using salmon gonadotropin releasing hormone analogue (sGnRH<sub>a</sub>) at dose of 250 ng/g BW, at 7 day intervals during the reproductive photoperiod produced final maturation after 3 injections. Spawning induction by using sGnRH<sub>a</sub> at dose of 1,000 ng/g BW was an effective technique than hydrogen peroxide ( $0.6 \times 10^{-2}\%$ ) and was considered the most practical method because no mortality occurred after spawning. Embryonic and larval development of *C. sandwicensis* occurred rapidly. The larvae hatched in 12-14 hr after fertilization and subsequently achieved metamorphic competence at two days old. Larval rearing was attempted to test the survival and possible settlement and metamorphosis on different diatoms and pelagic algae but failed at Day 9. We speculate that larval mortality after Day 9 could be caused by unsuitable benthic diatoms. This led to the incomplete life cycle of the opihi in the laboratory, due to mortality of the settled larvae. More work is needed to determine if our maturation methods are truly effective, on spawning techniques to improve seed production and larval rearing in order to complete the life cycle of opihi to make sustainable aquaculture of opihi possible in the future.

## Table of contents

DEDICATION .....	iii
ACKNOWLEDGMENTS .....	iv
ABSTRACT .....	vi
Table of contents .....	viii
List of Tables .....	xi
List of Figures .....	xiii
List of Abbreviations .....	xv
List of Publications .....	xvi
INTRODUCTION .....	1
CHAPTER 1 . COLLECTION, HANDLING AND ESTABLISHING HOLDING SYSTEM FOR OPIHI .....	5
INTRODUCTION.....	5
MATERIALS AND METHODS .....	7
Collection and transportation to the laboratory .....	7
Maintenance of animal .....	7
RESULTS.....	8
Collecting animals alive .....	8
Transfer mortality .....	10
Mortality of animal due to weighing .....	11
DISCUSSION .....	12
CHAPTER 2 . NATURAL FEED AND FEEDING BEHAVIOR ON BIOFILM OF OPIHI.....	15
INTRODUCTION.....	15
MATERIALS AND METHODS .....	16
Stomach content analysis .....	16
Growth of biofilm on various plastic substrates.....	16
Biofilm characterization .....	17
Feeding of opihi on biofilm.....	17
Calculating the feeding rate of opihi on biofilm.....	17
Soft tissue weight of opihi.....	18
RESULTS.....	18
Stomach content analysis .....	18
Typical opihi stomach contents .....	19
Biofilm characterization .....	22
Feeding on biofilm .....	23
DISCUSSION .....	25
CHAPTER 3 . DEVELOPMENT OF ARTIFICIAL DIET FOR OPIHI: FEEDING PREFERENCES .....	28
INTRODUCTION.....	28
MATERIALS AND METHODS .....	32
Preliminary feeding preferences on sea urchin feed.....	32
Experimental animals .....	32
Feed ingredients palatability and additional attractants tests .....	33
Growth performance of opihi on artificial feed.....	38
Experimental animals .....	39
Dietary preparation .....	40

Data analysis.....	40
Water stability test.....	40
<b>RESULTS.....</b>	<b>42</b>
Preliminary test with sea urchin feed .....	42
Feed ingredients palatability and additional attractants test.....	43
Growth performance of opihi during final trials .....	48
<b>DISCUSSION .....</b>	<b>52</b>
<b>CHAPTER 4 . THE EFFECT OF DIETARY PROTEIN LEVEL ON GROWTH</b>	
<b>PERFORMANCE OF OPIHI .....</b>	<b>57</b>
<b>INTRODUCTION.....</b>	<b>57</b>
<b>MATERIALS AND METHODS .....</b>	<b>63</b>
Experimental diets .....	63
Experimental animals .....	66
Data analysis.....	68
<b>RESULTS.....</b>	<b>68</b>
<b>DISCUSSION .....</b>	<b>77</b>
<b>CHAPTER 5 . THE EFFECT OF DIETARY CARBOHYDRATE LEVEL ON GROWTH</b>	
<b>PERFORMANCE OF OPIHI .....</b>	<b>81</b>
<b>INTRODUCTION.....</b>	<b>81</b>
<b>MATERIALS AND METHODS .....</b>	<b>83</b>
Experimental diets .....	83
Gelatinization.....	84
Experimental animals .....	85
Water stability test.....	86
Statistical analysis .....	86
<b>RESULTS.....</b>	<b>86</b>
<b>DISCUSSION .....</b>	<b>96</b>
In conclusion .....	99
<b>CHAPTER 6 . REPRODUCTIVE PERFORMANCE, FINAL MATURATION AND</b>	
<b>SPAWNING INDUCTION OF OPIHI .....</b>	<b>102</b>
<b>INTRODUCTION.....</b>	<b>102</b>
<b>MATERIALS AND METHODS .....</b>	<b>110</b>
Spawning season determination .....	110
Maturation trials .....	110
Spawning induction trials .....	116
Ripe broodstock definitions.....	116
Induction of spawning by using hydrogen peroxide .....	118
Induction of spawning by using sGnRH $\alpha$ .....	119
Handling of eggs.....	120
Fertilization and hatching determination.....	120
Handling of larvae .....	121
Algae preparation for larval settlement .....	121
Larval settlement aquaria preparation .....	123
Experimental larvae.....	125
<b>RESULTS.....</b>	<b>126</b>
Spawning season .....	126

Induction of final maturation.....	134
Hormonal induction of final maturation.....	139
Spawning induction by using H <sub>2</sub> O <sub>2</sub> .....	140
Spawning induction by using sGnRH $\alpha$ .....	142
Embryonic and larval development.....	143
Larval settlement trials .....	149
DISCUSSION .....	153
LITERATURES CITED.....	162

## List of Tables

Table 1.1. Mortality of opihi due to capture in all trials.....	9
Table 1.2. Mortality of animal due to tank to tank transfer. ....	10
Table 1.3. Mortality of animals due to handling during feed trial.....	11
Table 2.1. List of identified diatoms species in gut content of opihi.....	19
Table 2.2. Growth of biofilm on different plastic sheets.....	21
Table 2.3. The % dry matter/body weight/day for opihi feeding on biofilm.....	23
Table 2.4. Weight of mass film.....	24
Table 3.1. Growth performance of abalone fed the different diets (Cho <i>et al.</i> , 2008).....	30
Table 3.2. Growth performance of abalone fed the different diets (Cho 2010).....	31
Table 3.3. Ingredients composition (% dry matter) of gel diets.....	34
Table 3.4. Ingredients composition (% dry matter) of diets with additional attractive. ....	36
Table 3.5. Ingredients composition (% dry matter) for final taste test diet. ....	37
Table 3.6. Ingredients (% dry matter) of the diets. ....	39
Table 3.7. The composition of diets with various agar levels. ....	41
Table 3.8. The composition of proposed diets with various alginate levels. ....	41
Table 3.9. Feed consumption of opihi fed sea urchin feeds.....	42
Table 3.10. Feed consumption of individual animal in each diet (n=3). ....	44
Table 3.11. Feed consumption of individual animals fed various diets.....	46
Table 3.12. Confirmatory trials for feed ingredient preferences and substitution biofilm ...	47
Table 3.13. Total body weights (g), SGR (% day <sup>-1</sup> ), shell length (cm), feed consumption. ....	49
Table 3.14. The A/E ratio amino acids profiles of grow out diets and opihi tissue.....	50
Table 4.1. Effect of protein levels on growth performance of abalone (Mai <i>et al.</i> , 1995). ..	58
Table 4.2. Weight gain of abalone fed various dietary protein levels (Coote <i>et al.</i> , 2000)..	60
Table 4.3. Weight gain of abalone fed various dietary protein levels (Sales <i>et al.</i> , 2003)..	61
Table 4.4. Growth performance of abalone fed different dietary protein (Britz <i>et al.</i> , 1996).61	
Table 4.5. Growth response of abalone to various protein:energy. ....	62
Table 4.6. A summary of the protein requirement study for abalone. ....	63
Table 4.7. Composition of formulated diet (% dry matter). ....	65
Table 5.1. Weight gain and feed conversion ratio of abalone fed various lipid/carbohydrate levels. ....	82
Table 5.2. Weight gain of abalone fed various carbohydrate sources. ....	83
Table 5.3. Composition of dietary carbohydrate level.....	84
Table 5.4. Weight (g), SGR (%BW/day), feed consumption (%DM/BW/day) measurement of animal fed dietary of 18% carbohydrate. ....	88
Table 5.5. Weight (g), SGR (%BW/day), feed consumption (%DM/BW/day) measurement of animal fed dietary of 27% carbohydrate. ....	89
Table 5.6. Weight (g), SGR (%BW/day), feed consumption (%DM/BW/day) of animal fed dietary of 32% carbohydrate. ....	90

Table 5.7. Weight, SGR, feed consumption (%DM/BW/day) measurement of animal fed dietary of 37 % carbohydrate. ....	91
Table 5.8. The growth performance of opihi fed various dietary carbohydrate levels; SGR, specific growth rate; feed consumption rate (%DM/BW/day). ....	93
Table 5.9. The Tukey Kramer analysis between the treatments at 95% confidence interval.	94
Table 5.10. Water stability test of two dietary carbohydrate levels.....	96
Table 5.11. Composition of formulated diet for .....	101
Table 6.1. The ARA/EPA ratio from several trials on maturation. ....	105
Table 6.2. Maturation stages of gonad of limpets (Corpuz 1983; McCarthy <i>et al.</i> , 2008). ....	108
Table 6.3. Composition of formulated diet used for initial mutation trial. ....	111
Table 6.4. Fatty acid composition of growout diet used for maturation trial. ....	112
Table 6.5. Composition of formulated diets with different ARA levels.....	114
Table 6.6. ARA and EPA analysis of control and low ARA diets .....	115
Table 6.7. Composition of formulated diet with various ARA levels. ....	115
Table 6.8. List of materials and equipments with an appropriate cleaning technique for algae culture.....	122
Table 6.9. F2 medium preparation for cultivation of algae. ....	123
Table 6.10. Average size and GSI of sampled opihi for the reproductive cycle. ....	127
Table 6.11. GSI of male and female opihi throughout the study. ....	130
Table 6.12. Representative potential fecundity of several mature opihi.....	133
Table 6.13. Gonadal somatic index and egg size of animals fed various ARA levels. ....	136
Table 6.14. The effect of various H <sub>2</sub> O <sub>2</sub> concentrations on spawning of opihi. ....	141
Table 6.15. Spawning trial using H <sub>2</sub> O <sub>2</sub> at 0.60 x 10 <sup>-2</sup> %.....	142
Table 6.16. Spawning trials using hormone sGnRHα.....	143
Table 6.17. Embryonic and larval development of opihi <i>C. sandwicensis</i> at (22°C). ....	145
Table 6.18. Benthic diatom and pelagic algae growth on plate-substrate. ....	150

## List of Figures

Figure 1.1. The memorials near the water edge at Blowhole lookout, Oahu .....	6
Figure 1.2. Rough water area at Kalapaki Circle, Lihue, Kauai .....	6
Figure 1.3. Mortality of animal due to collection in all trials .....	9
Figure 1.4. A circular holding biofilm tank without plastic .....	10
Figure 1.5. Experimental colander with an opihi on it; a small square is a piece of feed ....	11
Figure 1.6. Soft plastic containers with both cylinder-shaped and cones-shaped .....	12
Figure 2.1. A representative aquarium liner by plastic sheet using for biofilm growth. ....	17
Figure 2.2. Several representative diatoms in stomach content of opihi .....	20
Figure 2.3. Unidentified particles, bacterial clumps (left) and thallus of macroalgae (right).	21
Figure 2.4. Diatom growth on rough plastic (A) and clear plastic (B) sheet at Day 7. ....	22
Figure 2.5. Representative biofilm growth on the plastic sheet.....	22
Figure 2.6. The microscopic (40x) benthic diatoms species in the biofilm aquarium.....	23
Figure 2.7. The clear circle area of biofilm was eaten by opihi.....	24
Figure 2.8. The relationship between the soft body weight and total body weight of opihi.	25
Figure 3.1. A difference between an opihi that eaten feed (left) and one that ate little or no feed (right). .....	43
Figure 3.2. Feed weight of various agar levels versus time immersing in seawater, n=3. ...	51
Figure 3.3. Feed weight of various alginate levels versus immersing in seawater.....	52
Figure 4.1. Relationship between weight gain and dietary protein level for abalone.....	59
Figure 5.1. Scatter plot of weight gain versus the total weight of animals in carbohydrate.	87
Figure 5.2. Relationship between normalized weight gain and dietary carbohydrate level .	95
Figure 5.3. Feed wet weight immersing in seawater by the time. ....	96
Figure 6.1. Classification of sexually mature male and female opihi.....	118
Figure 6.2. Experimental diatoms culture aquaria under shade area. ....	125
Figure 6.3. Seasonal changes in GSI of opihi <i>C. sandwicensis</i> . ....	128
Figure 6.4. Seasonal changes in GSI of males (n = 7-19) and females (n = 8-14) opihi....	129
Figure 6.5. Cross-sections showing stages of opihi gonad development. ....	132
Figure 6.6. Monthly distribution of sexes of <i>C. sandwicensis</i> .....	134
Figure 6.7. Monthly GSI change of broodstock (n=3) fed with growout diet.....	135
Figure 6.8. Gonadal somatic index of opihi <i>C. sandwicensis</i> fed various ARA diets. ....	137
Figure 6.9. Cross-section ovary of opihi <i>C. sandwicensis</i> stained with hematoxylin-eosin.	138
Figure 6.10. Gonadosomatic growth of opihi by hormone induction.....	139
Figure 6.11. The effect of hormones on gonad maturation (hematoxylin-eosin staining). .	140
Figure 6.12. Embryonic development stage of opihi <i>C. sandwicensis</i> .....	146
Figure 6.13. Embryonic development stage of opihi <i>C. sandwicensis</i> . ....	147
Figure 6.14. Embryonic and larval development stage of opihi <i>C. sandwicensis</i> . ....	148
Figure 6.15. Larval development stage of opihi <i>C. sandwicensis</i> . ....	149

Figure 6.16. Benthic diatoms and pelagic algae growth on settlement substrate ..... 150  
Figure 6.17. Metamorphosed larvae on different combinations of benthic diatoms and pelagic  
algae ..... 152  
Figure 6.18. Free swimming larvae day 2 (left) and settled larvae fed on diatom in day 4.153  
Figure 6.19. Temporal changes in water temperature and daylength in Hawaii. .... 157

## List of Abbreviations

GSI	:	Gonadosomatic index
SD	:	Standard deviation
min	:	Minute
hr	:	Hour
ppt	:	Part per thousand
m	:	Meter
cm	:	Centimeter
mm	:	Millimeter
$\mu\text{m}$		Micrometer
L	:	Liter
mL	:	Milliliter
$\mu\text{l}$		Microliter
$\mu\text{M}$		Micromole
kg	:	Kilogram
g	:	Gram
mg	:	Milligram
$\mu\text{g}$	:	Microgram
ng	:	Nanogram
ppt	:	Parts per thousand
%	:	Percent
$^{\circ}\text{C}$	:	Degree of Celsius
GnRH	:	Gonadotrophin releasing hormone
ARA	:	Arachidonic acid
EPA	:	Eicosapentaenoic acid
GABA	:	Gamma aminobutyric acid
DMPT	:	Dimethyl propiothetin

## List of Publications

Hua, T.N. and Ako, H. 2014. Reproductive Biology and Effect of Arachidonic Acid Level in Broodstock Diet on Final Maturation of the Hawaiian Limpet *Cellana sandwicensis*. Journal of Aquaculture research and development. *Manuscript accepted*.

Hua, T.N. and Ako, H. 2014. Effects of dietary protein and carbohydrate levels on growth performance of the Hawaiian limpet *Cellana sandwicensis*. Manuscript submitted to the journal Aquaculture Nutrition.

Hua, T.N. and Ako, H. 2012. Enabling studies for aquaculture of the Hawaiian Opihi, the Limpet *Cellana*. World Aquaculture Society. 12: 40-44

Hua, T.N. and Ako, H. 2013. Maturation and spawning induction in Hawaiian opihi *Cellana* spp. by hormone GnRH. Larvi'13-Fish & shellfish larviculture symposium. C.I. Hendry (Editor). European Aquaculture Society. 194-197.

### List of un-publications

Hua, T. N. and Ako, H. 2012. Development of aquaculture technology for opihi. center for tropical and subtropical aquaculture. Regional-E-notes. August 2012, Vol 4. Issue 8.

### List of oral and poster presentations

Hua. T. N. and Ako, H. Development of artificial diets for Hawaiian limpet *Cellana* spp. Presentation at the World Aquaculture Meeting 2013. Tennessee. February 21-15, 2013.

Hua. T. N. and Ako, H. Spawning and embryonic development of opihi *cellana sandwicensis*. 25<sup>th</sup> Annual CTAHR and COE Student Research Symposium; April 12-13, 2013. University of Hawaii at Manoa.

Ako, H., and Nhan, T.H. 2012. Initial studies on the aquaculture of the hawaiian opihi *Cellana*. 24<sup>th</sup> Annual CTAHR and COE Student Research Symposium; April 13-14. University of Hawaii at Manoa.

Ako, H. and N. Hua. 2011. Aquaculture of the Giant Opihi. Development of an artificial feed. The Biofuels Co-products workshop, Oceanic Institute. December 1<sup>st</sup>-2<sup>nd</sup>.

Ako, H. and N. Hua. Aquaculture of the Giant Opihi. Presentation at the joint CTAHR/Hawaii Aquaculture and Aquaponics Society, Windward Community College, Honolulu, Hawaii. November 12, 2011.

## INTRODUCTION

Our purpose was to develop appropriate commercial aquaculture technique for Hawaiian limpet known as opihi in Hawai'i. Three are main endemic Hawaiian limpet species including black foot or makaiauli (*Cellana exarata*), yellow foot or ālinalina (*C. sandwicensis*), and the largest species, giant opihi or kō'ele (*C. talcosa*) (Kay and Magruder 1977). They occupy different vertical zones; *C. exarata* exhibits at higher intertidal zones which are subject to the most extreme temperatures and desiccating conditions; *C. sandwicensis* is typically exposed to low intertidal zones with washed by waves and rarely submerged or exposed by tide and *C. talcosa* reside at the low intertidal to shallow subtidal shoreline (Kay and Magruder 1977; Corpuz 1981; Bird *et al.*, 2007; Bird 2011).

Opihi are considered one of the potential candidate species among the Hawaiian seafoods for commercial aquaculture development because of their high value food market. From a commercial perspective, there is a high and increasing demand for opihi in Hawaii because they have been harvested by Hawaiians for food for centuries. The intensive harvesting for private consumption and commercially have led to overharvesting. A major commercial catch decreased significantly from 150,000 pounds in the 1900s to about 10,000 pounds in 1978 (Iacchei 2011). The scarcity has driven up prices to about \$200/gallon shell on (Thompson 2011).

Opihi are also culturally important in Hawaiian society. For example, per communication with Nelson Ka'ai (opih picker, Kauai), he said that many people readily paid me or other opihi pickers with the high prices to pick opihi from the wild just to serve at cultural important events, birthday parties or family gathering. Among the three species, yellow and giant opihi are the most prefer. In addition to an important food sources, opihi's shell also continue to be used as tools for scraping skin off taro plant, sweet potato and grating the coconut meat before eating (Handy *et al.*, 1991) and as decorative elements in jewelry.

It is dangerous to collect opihi. It has been long recognized by the Hawaiians called them "He i'a make ka opihi" which means that the opihi is a fish of death (Thompson 2011 cited Pukui 1983). This reflects the dangers of gathering picking opihi. Opihi pickers are swept out to

the sea and drown while collecting opihi every year. Monuments of opihi pickers have had been created in most of collecting sites.

Several previous preliminary studies showed that juvenile opihi *C. sandwicensis* grow fast in the wild, but they grew at different growth rates (Corpuz 1983, Bird 2006). It is possibly due to variety of food sources by seasons. Iacchei (2012) reported that opihi could reach 3.2 cm shell length in seven months after settlement in the wild. Our studies will show that opihi could reach to the market size (assumed to 3.5-4.0 cm, shell length) in about 12 months starting at 0.5 cm shell length. We will show that opihi do not need a sophisticated culture system as previous studies thought. Thus, these characterizes indicate the feasibility of high reproduction among commercially cultured opihi.

In this dissertation, a variety of experiments have been employed hoping to investigate the aquaculture technologies for opihi farming in Hawai'i Islands. The first chapter will focus on establishing the optimal handling system to hold the animal alive while in the laboratory. This is a challenge due to high capture and transfer mortalities that were observed when first working with opihi. Initial mortalities during capture were about 58 % and another 42 % transfer mortalities was observed when remove them from holding tank to experimental aquaria. We will describe how we learned to validate the use of plastic linings in aquaria or house animals in plastic colanders or soft containers. These methods make removal from one container to another without killing the animals possible. It is less dangerous but mortality during transfer or handling for weighing remains the main cause of death. Eventually we used soft container as a good holder facility that completely solved removal mortality.

The second chapter will focus on feeding on natural feeds. The stomach content of opihi microscopy was first determined to examine what they eat in the wild. We will describe the growing of the natural diatoms we will call biofilm in the laboratory and how we learned to feed opihi on it as a means of holding animals between feed trials. However, these natural feeds have to be cultured, and travel to and from the sea side growing site is inconvenient to pick up fresh samples. Thus, a study of artificial feed was determined to be necessary.

The third chapter will focus on development of an artificial feed. There have been no studies on artificial feed for opihi to date. There have been intensive studied on artificial feed for abalone, a close gastropod species to opihi. Literature studies on the development of abalone feed will be discussed in detail in next chapter. The aquaculture of opihi needs to have a reliable

commercial feed available. Feed palatability consideration was found to be an important consideration in animal growth relationship, and therefore was made the first priority. We began our studies with several preliminary tests with sea urchin feeds. Several chemical attractants were tested including betaine, gamma aminobutyric acid (GABA), dimethyl propiothetin (DMPT), biofilm incorporation as feed attractant. First we found that biofilm was a necessary attractant. Eventually we found that *Porphyra* preparations could replace biofilm as a key feeding stimulant. This algal is known as red algal *Porphyra yezoensis* or *P. tenera*. It sold as Nori or Laver and has wide commercial available.

In the following study, literature review found the potential ingredients consisting of casein, fishmeal, soybean meal, krill meal, shrimp head meal and green tea by product. However, shrimp head meal is of obvious concern and not really well recommended in Hawai'i due to the possibility that it contains shrimp viruses such as whitespot virus. Another ingredient is green tea by product which is not readily available. Casein diet is also a promising ingredient but it is too expensive to try. We found that diets containing fish meal and soybean meal were moderately palatable but adding krill meal to this boosted palatability so that feeds were eaten at >1% of bodyweight per day. We then focused on the nutritional properties of the diet.

In chapter 4, our tests of dietary protein requirements for optimal growth are detailed. We followed the hypothesis of matching amino acid profile feed with amino acid of animal tissue was first proposed by Ako and Dominy (1987). We found that there was no significant difference in weight gain of those opihi that were fed dietary protein levels ranging from 27 % to 47 % in the first protein trial. It may be due to low survival rates among the diets when removing animals from colander for weighing. However, better and significant data were obtained from the second protein trial when the animals were fed with dietary protein ranging from 21 % to 50 %. Statistical analysis showed that there was a significant difference in weight gain and specific growth rates of animals among the diets. The polynomial regression analysis revealed there is a trend of increasing growth rate with increasing dietary protein up to 35 % and reduced significantly at the 50 % diet.

Our tests of different carbohydrate levels for the optimum growth performance of opihi are covered in chapter 5. The results show that there was a significant difference in growth performances of opihi that were fed different dietary carbohydrate levels ranging from 18% to 37%. Among of those tested carbohydrate diets, the growth response of opihi fed 27 % and 32

% diets was significantly higher than those animals that were fed with 18% and 37% carbohydrate diets. A 32 % carbohydrate level would be recommended as it is not an expensive feed stuff compared to non-nutrient ingredients.

Our investigation of the characteristics of feed palatability and of nutrient requirements has given us sufficient information that we have been able to generate an effective commercial feed for the aquaculture of opihi and have fed to animals.

The sixth chapter will describe the reproductive performance of opihi in the wild, investigation of optimal conditions for final maturation in the laboratory, induction of spawning and larvae rearing in the laboratory. The spawning cycle of opihi *C. sandwicensis* was determined by monthly collection GSI of wild animals for a whole year. The data suggested that the peak spawning season of opihi would occur from November to December and could be extended to January. Non-reproductive season was proposed to take place from late February to early September and followed by a final maturation phase which occurred in October. An attempt was made to mature animals in the laboratory using a maturation diet incorporating different arachidonic acid levels in diets, which contained the same ARA to EPA ratio. In addition, it was found that the broodstock opihi could be matured by application of sGnRH $\alpha$  at low level (250 ng/g body weight) in the laboratory.

Two different spawning methods including hydrogen peroxide and sGnRH $\alpha$  were used to obtain the optimal spawning method. We will show that hydrogen peroxide is toxic and has a non-specific effect on broodstock therefore it is not a recommended technique for spawning induction in opihi. Induction of spawning by using GnRH is the preferred technique and may consider as the practical method because no mortalities occurred after injection. The completed embryonic and larval development processes of *C. sandwicensis* were observed in this study. Embryonic development occurred rapidly and hatch-out occurred in 12-14 hr after spawning. Lastly, an attempting larval rearing was also tried to test the survival and possible settlement and metamorphosis on different benthic diatoms and pelagic algae will be also covered in this chapter.

## CHAPTER 1 . COLLECTION, HANDLING AND ESTABLISHING HOLDING SYSTEM FOR OPIHI

### INTRODUCTION

Wild specimens have to be collected alive for experimental purposes until such time as animals can be produced in the laboratory. Learning how to collect animal with minimal mortality and subsequently knowing how to handle animals alive in captivity without killing them are essential aims in the present chapter.

While people have been collected opihi for food for centuries but they have not been keeping them alive. They simply eat them shortly after capture. Collecting and holding opihi alive is not a common practice. In modern times, it was widely believed that a sophisticated aquaculture system was needed to maintain a captive population. Corpuz (1983) raised opihi on vertical dishes sprayed with seawater periodically while Christopher Bird (personal communication) raised opihi in fill and dump tanks. These sorts of facilities would be too expensive to construct and operate for a practical commercial aquaculture operation.

We were the first to recognize the difficulty of collecting and maintaining opihi for biological studies. Natural ecological distribution of opihi in different intertidal zones gives the different species of opihi survival advantages. The three kinds of opihi have very different zones of habitation on rocky shores. The koele *C. talcosa* live in deep water and not on wave splashed rocks like the other species. This raises the possibility that they may be easily grown in tanks rather than in a sophisticated set up. When we first worked with this opihi species, Chris Bird had to dive in about 10 feet of water to collect giant opihi. He had to requisite water skills to make this relatively safe. In addition, according to Chris Bird (2006), said that there is a scarcity of giant opihi in many areas, especially in Oahu. For this reason, Nhan Hua decided to switch studies to the yellow foot opihi, *C. sandwicensis* which can be collected during low tide by moving on the rocks. There is less risk of being drowned, but he always needed to keep an eye out to prevent himself from being swept away by the waves and drowning. Figure 1.1 shows an example of the memorials in traditional style assembled by Hawaiians for their relatives who were swept away and drowned while picking opihi at the edge of shoreline.



**Figure 1.1.** The memorials near the water edge at Blowhole lookout, Oahu, to opihi picker's death while picking opihi. Just steps from the site where opihi pickers were swept to the Ocean and drowned.

The rough water area where opihi must be collected is shown in the Figure 1.2.



**Figure 1.2.** Rough water area at Kalapaki Circle, Lihue, Kauai

The first challenge in collecting opihi alive was observed with the high mortalities in the first collection. There was 58% mortality. We believe this was due to injuries to animals while collecting them. It was hypothesized that minimizing mortality could be done via the skill of the collector while detaching them from the rocks. With experience, we were able eventually to collect animals with about 70 % survival.

Transfer mortality is another challenge because the animals cling tightly to the tank walls. It was hard to get them off the wall without further injury. The results will show that another 42% additional mortality occurred in the first transfer from the naked tank wall to another tank. Later we found that putting plastic sheets as tank liners solve this problem.

We also experimented with soft plastic colanders because we thought that we would remove animal as easily as with plastic sheets. However, mortality during weighing in feed trial was seen on hindsight and eventually we concluded that plastic colanders are not effective. Eventually, other soft containers were used. They could be easily flexed and animals would be removed free of mortality.

## **MATERIALS AND METHODS**

Collection and transportation to the laboratory. Opihi were collected from remote areas around the State of Hawaii. Nine capture trials were done in this study. The first collection was conducted in Kona Bay, Hawaii Island. Collected animals were put in a net and covered with a wet towel in a cooler and then transported to Oahu by air cargo. Upon arrival they were put in the holding tank. Other trials were done on Kauai Island with help from an experienced local collector and several other trials were done on Oahu. The animals that were collected in Oahu Island were transported the laboratory by car. They were placed into a plastic bag in a cooler to avoid attachment to the cooler wall.

Maintenance of animal. For the first collection, animals were stored in a holding biofilm tank (500 L), which was a naked tank without plastic liner. This will be shown later. This holding tank had biofilm growth in it. The tank was exposed to the sunlight with flowing seawater (about 30L/min) and aeration. This biofilm tank was prepared for a week prior to collection and removal of animals from this tank was problematic. Subsequent holdings were in aquaria with sheet plastic (polyethylene plastic, 4 mil) liners covered with biofilm. Different

plastic substrates were also employed to line in biofilm aquaria and naturally recruited biofilm will be described more detail in the next chapter.

Behavioral response and mortality of animals in each collection trial were monitored over the course of experiment. The opihi that could not attach to the biofilm tank walls were considered to be dead. The ones that were able to cling to the tank walls were considered to have minor injuries or without injury. It is noted that the mortality occurring immediately or up to Day 2 after transferring from holding biofilm tank to bioassay aquaria as considering transfer mortality.

Animals were removed easily by deforming the plastic sheet. Later soft plastic colanders (20 cm diameter) were used to house animal for feed trials and mortality due to removal off colander for weighing purposes were also recorded. Eventually soft plastic container was made by plastic screen with an inside layer soft plastic tubs was also tried to replace colander.

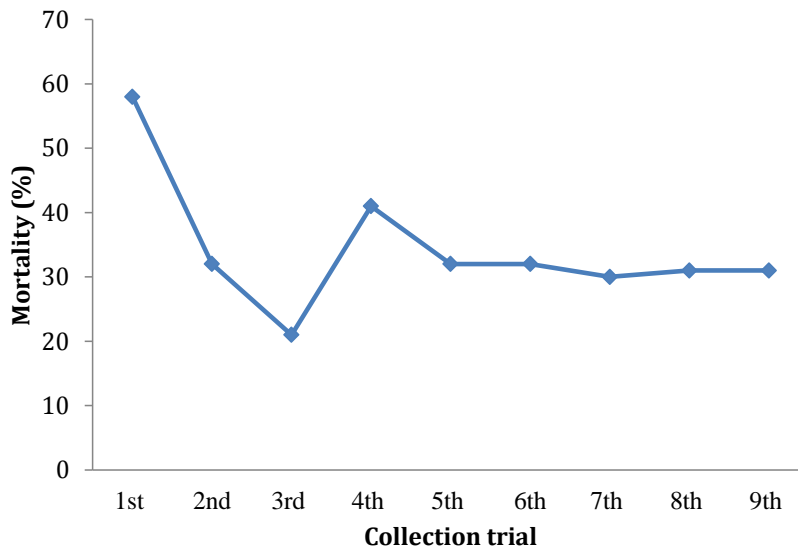
## **RESULTS**

Collecting animals alive. Mortality of collected animals in all captures was monitored in Table 1.1. The first collection was at Kona on Hawaii Island, where 40 animals were collected. Animals were scraped off the rocks in about 10 feet of water by Dr. Bird, a postdoctoral at HIMB. Giant opihi were found in vertical sides of the rocks. They were scraped off with an oyster shucker or putty knife, which consist of a thin metal blade about 1 inch across and 1 mm thick. This was inserted between rock and foot of opihi. We tried to scrape them off before they got alarmed and clung more tightly to the rocks. Within 4 days, a total of 23 animals died. The mortality was decreased to 16 out of 49 animals in the second collection (Kauai), with about a 30% mortality rate in subsequent collections (5-9) from Oahu. This presumably was due to increased experience of the collector and greater care taken during collection. In general, mortality was observed mostly on the first two days, with fewer on Days 3 and 4 after capture. It was assumed that mortalities were due to injury during capture which we could not detect by physical examination.

**Table 1.1.** Mortality of opihi due to capture in all trials.

Site of collection	Kona			Kauai			Oahu		
Collection trial	1	2	3	4	5	6	7	8	9
No. of collected animals	40	49	33	34	25	22	27	29	16
Mortality:									
Day 1	5	4	3	10	4	3	4	5	3
Day 2	5	10	2	4	2	2	2	2	2
Day 3	5	0	2	0	2	2	0	2	0
Day 4	8	2	0	0	0	0	2	0	0

Figure 1.3 shows the mortality in percentages of animal in all collections. Mortality seemed to constant at the same rate from the fifth collection. It seems that a 30 % mortality may be accomplished with practice. However, this could be underestimated as we have found out that a total of 59 % mortality for the last three collection trials for the second protein trial. It is noticed that these mortalities occurred after collection and hold them in aquarium without algae seedless from Hawaii Institute for Marine Biology (HIMB). They were used right after the second day after collection.



**Figure 1.3.** Mortality of animal due to collection in all trials

Transfer mortality. Table 1.2 shows the mortalities of animals when they were transferred from holding biofilm tank to bioassay aquaria. The worst mortality occurred when the 17 survivors of the first collection trial were transferred from the naked tank (circular tank without plastic liner, Fig. 1.4) to experimental aquaria. The mortality was 42% (7 animals). The mortality was reduced from the second transfer the survivors of the second collection and on. This was because the animals were transferred from plastic lined aquaria to the plastic lined bioassay aquaria. As stated previously, animals were removed easily from plastic substrate by simply deforming or scraping off the plastic substrate.

**Table 1.2.** Mortality of animal due to tank to tank transfer

Collection trial	1	2	3	4	5	6	7	8	9
No. of animals	17	33	26	20	17	15	19	21	11
Transfer from	Naked tank to bioassay aquaria		Plastic lined aquaria to bioassay aquaria						
Transfer mortality (ind.)	7	1	3	0	0	0	0	0	0



**Figure 1.4.** A circular holding biofilm tank without plastic liner, and three aquaria with plastic sheer liner above, used for the second and following holdings.

Mortality of animal due to weighing. We settled on colanders to house animals during feeding trials. Table 1.3 shows there were 21% and 17% mortalities due to injury during weighing for both protein and carbohydrate trials respectively. Animals were weighed only three times and there were as many mortalities as during the other 90 days of the trials. The mortalities occurred right after removing them from the colander's wall. It is speculated that the mortalities were due to muscle injury by laboratory spatula. The data indicate that colanders are not a suitable facility for holding opihi (Fig. 1.5).

**Table 1.3.** Mortality of animals due to handling during feed trial

Feed trial	No. of animals	Mortality (ind.)	
		Weighing	unknown
Protein (trial 1)	43	9	8
Carbohydrate	36	6	4



**Figure 1.5.** Experimental colander with an opihi on it; a small square is a piece of feed

We then replaced the colanders with containers made of soft plastic screen. The soft containers were held in layers plastic tubs. Our preliminary observation found that the animals

were healthy and fed well while they were held in soft container (Fig. 1.6). The animals were removed easily by distorting the screen plastic without any mortality. The soft containers were with layer plastic tubs.



**Figure 1.6.** Soft plastic containers with both cylinder-shaped and cones-shaped for holding animal

## DISCUSSION

The first step in this study involved the collection and handling of wild animals to be used in feeding trials and to later serve as breeding stock. We needed to have live and healthy animals for developing feed trial. Our original thought that the giant opihi would hold a potential aquaculture in Hawai‘i because it is the biggest opihi and was reported to grow faster than other species (Kay *et al.*, 2006). This species was also high rate flavor and is preferred as much as the yellow opihi (Bird 2006). Another aspect of the feasibility of aquaculture of this species was relative to its performance for living below the waterline. This may suggest a regular aquaculture system operation. However, when we were in the first collection trial, we recognized that it was too dangerous for the graduate student to collect them at the shoreline water. According to Bird (2011), the giant opihi is only found in the main Hawaiian Islands, from Hawai‘i to Ni‘ihau. Thus, our target study species was then switched to the yellow foot opihi *C. sandwicensis*.

Our study was the first to reveal that the yellow foot opihi was healthy and fed well in the experimental aquaria without intermittent water sprayed, or dump tanks. This was in spite of their naturally rocky habitat washed by waves on intertidal zones (Kay and Magruder 1977, Bird

2007). The holding system of opihi in our study was much simpler. The opihi were held in aquaria with a water movement during feed trials. We don't know whether the movement of water in the handling system neither was necessary. We used a biofilter to remove large solids in the water and this is an inappropriate use of a biofilter. Previous studies reported that the function of respiratory in limpet is water movement dependent (Kington's 1968 in Branch 1981). They also reported that water flow through mantle cavity allows oxygen exchange. We used moving water and did not test stagnant water. We wonder how important and fast water movement is needed.

We discovered that dealing with live opihi involve challenges that are overcome. The first challenge is high mortality due to collection problem. We found that we could decrease mortality to 30% by being careful. Animals were fine in pre-conditioned biofilm tank and fed on biofilm before being used for other experiments. We attempted to observe muscle and mantle damaged but it was only seen in some cases. It is believed that animals with serious cuts died immediately after arrival the laboratory and the ones that with minor wounds would survive for a few days, a dissecting scope might have been useful. Similar observation have been made in abalone, they often succumb to wound suffered during removal off the substrates. Abalone blood has no clotting ability and relatively minor cut can cause death due to loss of haemolymph (Cox, 1962).

Rocky habitat and attachment to the substrates are factors. Opihi adhere to the substrate in washing rocks in the wild. They stick to holding tank with their muscular foot. When touched, the opihi used their foot muscle to pull shell down tightly against the substrate for protection. When opihi are in this defensive position it was difficult to detach them from the wall, and the more we touch them the more they cling tightly. It suggests that physical damage could happen while trying removal them off substrates.

Capture mortalities were reduced significantly to 33% and 21% in the second and third collections respectively, with an experienced opihi picker, Nelson Ka'ai. The reduced mortality indicates that we should pay attention and improve experiences for this work. Great care was taken to avoid muscle damage during removal off the rocks in subsequent captures. Eventually, capture mortality rate seems to be constant in about 30% in practice from the fifth collection and on. Thus, collecting experience is probably important but was not objectively proved in this work.

Plastic tank liners were our breakthrough for transferring animals from one tank to another. The worst transfer mortality (42%, among the survivors) occurred when removal animal from holding naked tank without plastic liners to experimental aquaria. Transfer mortality was decreased to zero or in very small percentage from the second trial through all the rest of trials when flexible plastic was used. Therefore, the matter of transfer mortality issue was solved with plastic aquaria liners. In addition, biofilm grows well on plastic substrates. More studied was done to observe growth rate of biofilm on and durability of different plastic substrate will be described detail in the next chapter.

We experimented housing animals in soft plastic colander for protein and carbohydrate levels (in the next chapters). We thought that removal animal from soft plastic colander for weighing would be similar the soft plastic liner and/or removal animal off colander substrate at night while they search for food would be harmless. This seemed to be the case for a while. However, weighing mortality occurred in the first protein and carbohydrate experiments were 21 and 17% respectively. This suggests that plastic colanders are not useful for holding animal in aquaculture system.

Another successful preliminary study used the thai gastropod, *Neothais harpa*. It is predator and when placed on the top of opihi, this predator causes opihi to try to flee. In this stage they could be removed from the tank walls on a card for transfer. We did not study this method extensively. However, we believed that the limitation of the method is that collectors do not have time to wait for reaction of opihi to the snail because the currents in the wild. This method can be useful in the laboratory conditions. However, it is also unvalued having to keep predator snails in the laboratory.

In conclusion. Although efforts have been tried to minimize the collection mortality and we could have approached about 30 % in general, however, the mortality due to collection and transfer seem to remain a problem area for the future. This fact can be solved until we would be able to reproduce animals technically in the hatchery.

## CHAPTER 2 . NATURAL FEED AND FEEDING BEHAVIOR ON BIOFILM OF OPIHI

### INTRODUCTION

Feeding preferences of opihi in the wild, learning how to culture and feed animals with natural feed were essential goals in the present chapter. One hope was to gain insight into the palatability that would be contributed to the development of a sustainable feed for opihi. Another goal was to have a feed to keep opihi alive in captivity until such time as an artificial feed could be developed.

Limpets display herbivore feeding behavior. They are believed to graze and scrape off microflora that naturally grows on rock surfaces by radula (Nicotri 1977, Steneck 1982). It is believed that where opihi live on the rocks, they feed naturally on benthic crustose coralline algae, cyanobacteria film in the field (Kay and Magruder 1977). However, much of the information is still limited and anecdotal and opihi's natural diet has not yet been well documented.

In this work we will examine the stomach contents of opihi microscopically to determine what they eat in the wild. The result will show that benthic diatoms were the most predominant identified materials in the stomach contents of opihi. We will also describe the laboratory culture of the natural feed (biofilm) in tanks on land and learning to feed opihi on it. It is also possible that biofilm would be a part of grow out feed for opihi because it was reported that live algae are still used along with artificial feed in commercial abalone *Haliotis midae* culture (Naidoo *et al.*, 2006). Our Hawaii abalone industry (the Big Island Abalone Co.) grows a Ezo abalone (*Haliotis discus hannai*) in part on natural feed. Natural feed including benthic diatoms are important principal food source for both larval and postlarval stages for grazer species (Hahn 1989; Kawamura *et al.*, 1998; Gordon *et al.*, 2006; Capipin 2007). These include larvae of mollusks species consisting of opihi, sea urchins and abalone, as well as a number of fish larvae and herbivorous adults.

Plastic sheet technology described in the previous chapter as a method for handling opihi without mortality was discovered here as a substrate for diatom growth. The experiment was also conducted to determine durable use in opihi handling system. The three different plastic sheets were used to test growth of a biofilm and to line aquaria.

This chapter was also described cultured biofilm formation. The results will show benthic diatoms were the most predominant materials in both wild animal gut content and artificial biofilm propagation. Our studies of cultured biofilm and opihi stomach contents will show that they are a mixture of benthic diatoms such as *Navicula*, *Nitzschia*, *Synedra*, *Melosira*, and so on, flagellates, ciliates (Euplotes types) as well as unidentifiable matter.

We had also observed the feeding rate of animals on the biofilm mat. The opihi ate biofilm at 0.47% drymatter per bodyweight per day. Biofilm was used for feeding opihi before and between feeding trials while they were used for testing of the developing artificial feed processes. The feeding level observed would serve as a recommended level for further feed studies.

## **MATERIALS AND METHODS**

Stomach content analysis. Feeding preferences of opihi in the wild was determined by the analysis of stomach contents. Ten opihi (5.0-8.5 cm shell length) that died immediately after collection were used for this study. They were frozen to interrupt gut content digestion. Each of animals was carefully dissected and each gut was sliced open, collecting its content which was then fixed in 5 mL of 4% formalin. Diatoms species and other particles were tentatively identified by inspection in comparison to photographs and description of diatoms of known species in books (Gilmartin and Revelante 1974; Saboski 1976; Lee 2008).

Growth of biofilm on various plastic substrates. Three different plastic sheets were used to line aquaria and naturally recruited biofilm was allowed to grow. The first one was sold as a dropcloth. It was a rough polyethylene plastic, 2 mil thick (Trimaco Durham NC, China). The second was a clear plastic sheet, 2 mil thick (Covalence plastics, Minneapolis, MN 55431, USA) and the third was a thicker polyethylene plastic (4 mil). The aquaria were supplied with open running seawater at a flow rate of approximately 3 Lmin<sup>-1</sup> that had been filtered with a sand filter and placed outdoors at HIMB (Fig. 2.1). The growth of biofilm in each aquarium was observed for 2 weeks by visual observation. Photographs were taken. The durability of each plastic was also attempted to monitor in this experiment.



**Figure 2.1.** A representative aquarium liner by plastic sheet using for biofilm growth.

Biofilm characterization. Three pieces ( $1.0 \text{ cm}^2/\text{piece}$ ) of biofilm mat were randomly collected in biofilm aquarium then put into sample bottles of 5 mL of 4 % formalin. They were swirled thoroughly by hand shake and then pipetted on to a microscope slide with a cover slip. Observation was with a compound microscope. Diatoms species and other particles were identified after examination under the microscope. These results will define as biofilm compositions or natural feed components in this study.

Feeding of opihi on biofilm. In order to evaluate feeding rate of opihi on natural feed, three aquaria were set up as described with naturally recruited biofilm, cultivated for two weeks prior to this test. Healthy animals were selected from holding aquaria. A total of 8 animals were used for this test, 2 or 3 animals were placed at various position in biofilm aquaria. This experiment was observed for 5 days. The amount of feed on biofilm was determined by estimating the area the animals cleaned up every day. The feeding amount was then converted to % dry matter per body weight per day, calculated as follow:

Calculating the feeding rate of opihi on biofilm. Biomass eaten/ $\text{cm}^2$  was calculated by randomly sampled three pieces of biofilm (4 cm x 3 cm; length x width; 3-4 mm thick) from the bottom and on the sides of the aquaria. These pieces were blotted on a towel paper to remove surface water. They were then weighed to yield wet weight. The dry weight of these mass were then obtained by drying in the oven at  $100 \text{ }^\circ\text{C}$  for 5 hr and weighing.

Soft tissue weight of opihi. To obtain the animal body weight (soft tissue), animal total weights were obtained by weighing and measuring animals that had recently been captured from the wild, had died, and had soft tissue dissected out and weighed. The relationship between the total weight and soft tissue weight was obtained when these data were plotted against each other on excel.

## **RESULTS**

Stomach content analysis. The gut contents of wild opihi which were examined under a compound microscope appeared to have mixture of benthic diatoms, bacterial clumps and other un-identifiable particles. About twenty diatom species which were identified (Table 2.1). They were listed in terms of estimated abundance. The diatom species comprised as much as about 30% (estimated based on the area covered by diatoms on glass slide under microscope at a certain volume) of the materials that could be identified by taking photos under a compound microscope in comparison to published photos.

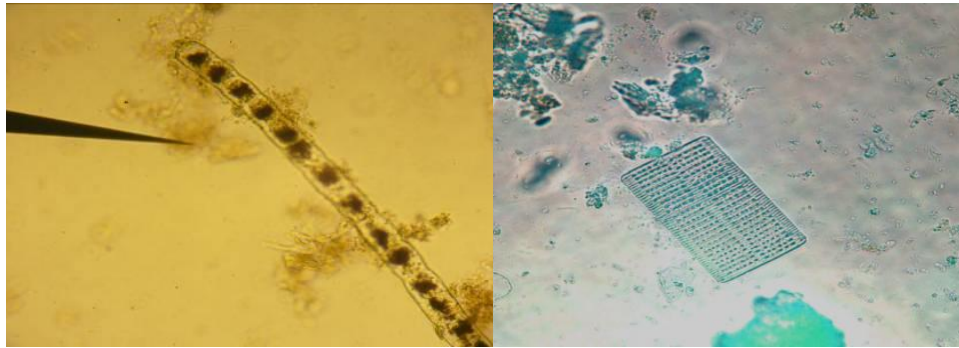
**Table 2.1.** List of identified diatoms species in gut content of opihi

Species	Dominant	Average	Appearance
<i>Bacillaria paxillifer</i>	+++		
<i>Fragilaria</i> sp.	+++		
<i>Melosira</i> sp.	+++		
<i>Navicula</i> sp.	+++		
<i>Rhabdonema</i> sp.	+++		
<i>Amphora</i> sp.		++	
<i>Climacosphenia</i> sp.		++	
<i>Grammatophora</i> sp.		++	
<i>Licmophora</i> sp.		++	
<i>Nitzchia</i> sp.		++	
<i>Pleurosigma</i> sp.		++	
<i>Tabellaria</i> sp.		++	
<i>Trigonium</i> sp.		++	
<i>Asterionella</i> sp.			+
<i>Cymbella</i> sp.			+
<i>Diplonesis</i> sp.			+
<i>Mastogloia</i> sp.			+
<i>Opephora</i> sp.			+
<i>Surirella</i> sp.			+
<i>Thalassionella</i> sp.			+

Notes: +++ indicates that the frequently abundant appearance in each animals;

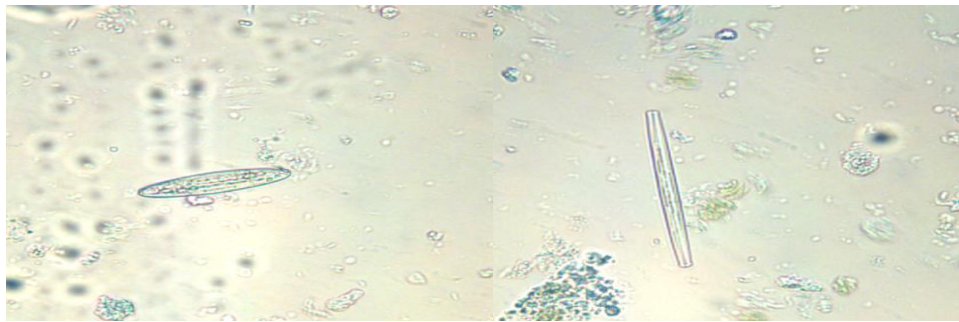
++frequently present in each animal; +appeared but less.

Typical opihi stomach contents. Several representative diatoms are shown in the Figure 2.2 along with unidentified particles (Fig. 2.3). Most frequently seen were the benthic diatoms *Bacillaria*, *Fragilaria*, *Melosira*, *Navicula*, and *Rabdonema*. Less often seen were *Amphora*, *Climacosphenia*, *Grammatophora*, *Licmophora*, *Nitzchia*, *Pleurosigma*, *Tabellaria*, and *Trigonium*. The results indicate that the opihi use the radula to scrape off and feed on benthic diatoms, bacteria and other matter that grown on surfaces of rocks.



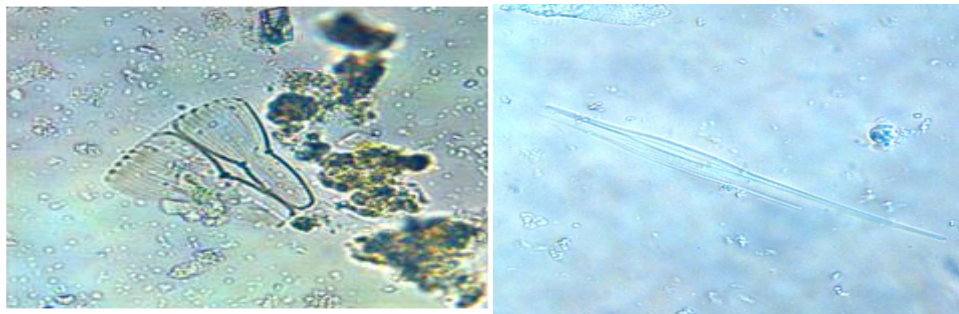
*Melosira* sp.

*Rhabdonema* sp.



*Navicula* sp.

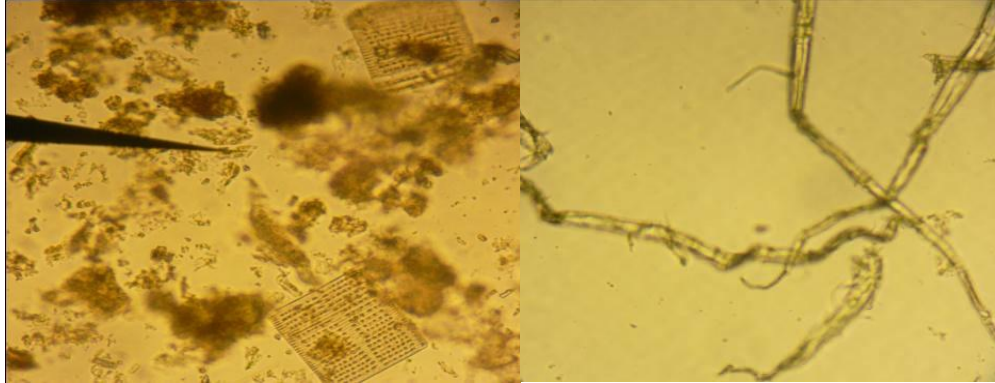
*Fragilaria* sp.



*Licmophora* sp.

*Nitzschia* sp.

**Figure 2.2.** Several representative diatoms in stomach content of opihi



**Figure 2.3.** Unidentified particles, bacterial clumps (left) and thallus of macroalgae (right).

Grow of biofilm on different plastic sheets is shown in Table 2.2. Biofilm grew spontaneously in aquaria when exposed to the sun and ocean spray. The result showed that the biofilm grown in a shorter number of days with rough plastic than a smooth plastic or thicker polyethylene plastic. It took only 7 days for Biofilm cover most of surface of the rough plastic, whereas it took about 10-14 days for a smooth plastic and thicker polyethylene plastic (Fig. 2.4). Salinity was maintained approximately 35 ppt, and the experiment was held under ambient temperature. The result showed that rough plastic to be superior to smooth plastic for biofilm growth. We therefore use rough plastic for our further experiments with biofilm and handling animals. In the other hand, thicker polyethylene plastic seems have better durability at the end of experiment and may be preferred for commercial use.

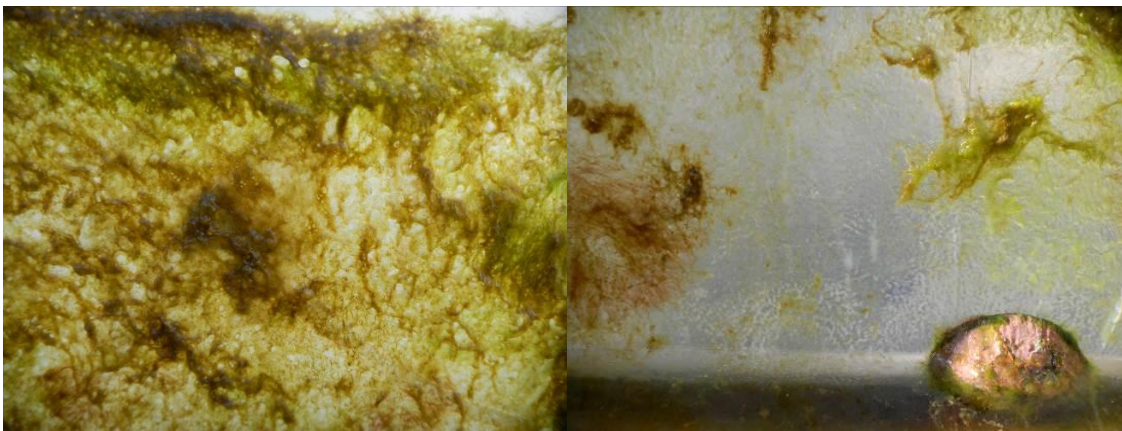
**Table 2.2.** Growth of biofilm on different plastic sheets

	Plastic type		
	Rough plastic (Dropcloth, 2 mil)	Clear plastic	Thicker polyethylene plastic
Algae growth in time (day)	7	10 -14	10-14
Durability (day)	60-70	60-90	90-120

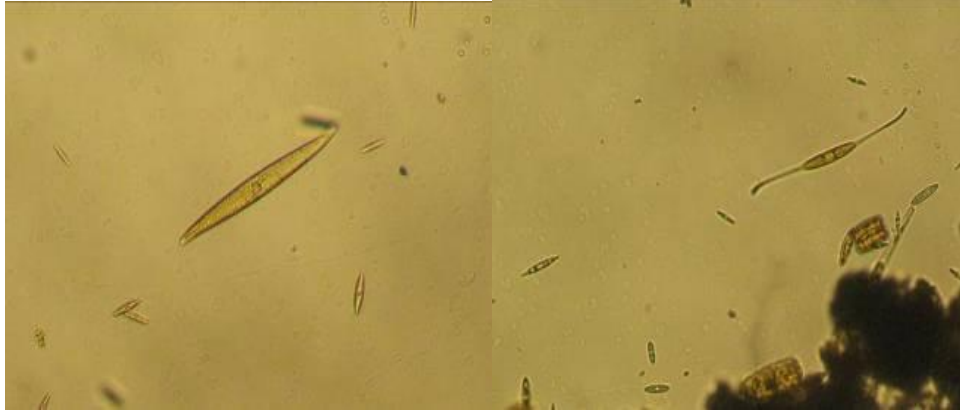


**Figure 2.4.** Diatom growth on rough plastic (A) and clear plastic (B) sheet at Day 7.

Biofilm characterization. The biofilm mat seemed to be dominated by a mixture of brown benthic microalgae in which assumed to be the assemblage of benthic diatoms, bacteria clumps, and newly settled sporelings and germlings of macroalgae such as green algae and red algae (Fig. 2.5). The brown benthic algae were observed under the compound microscope and diatoms species were identified (Fig. 2.6). Frequently seen diatoms were *Nitzschia*, *Rhizosolenia*, *Melosira*, *Coscinodiscus* and *Navicula*. Beside the benthic diatoms, the green algae *Ulva* and red algae *Polysiphonia sp* were also present in the biofilm aquarium.



**Figure 2.5.** Representative biofilm growth on the plastic sheet before feeding (left) and after feeding (right) by opihi.



**Figure 2.6.** The microscopic (40x) benthic diatoms species in the biofilm aquarium. Note probable bacterial clump on bottom right.

Feeding on biofilm. Biofilm is a close analogue to natural feed and opihi were observed to feed when placed in tanks. The mean feeding rate was 0.47% DM/BW/day (Table 2.3). Most of the animals ate frequently (77% of the nights) and two fed every day. Biofilm was consumed in varying amounts. The average of biofilm consumption was 0.30-0.66% DM/BW/day. Differences in feeding rate were probably due to overlapping feed area. Animals fed on area in which they were placed (Fig. 2.7). This feeding rate could be a reference level for further feed trial study, but we do not know what the natural optimal feeding rate for opihi in the field is. However, the amounts eaten and frequency of feeding have been documented here.

**Table 2.3.** The % dry matter/body weight/day for opihi feeding on biofilm

Observation day	No. of animals							
	1	2	3	4	5	6	7	8
Day 1	0.70	0.59	1.30	0.00	0.00	1.20	0.25	0.00
Day 2	0.93	0.60	0.00	0.00	0.84	0.00	0.30	0.00
Day 3	0.12	0.42	0.42	0.74	0.78	0.00	0.87	0.75
Day 4	0.00	1.12	0.55	0.00	0.00	0.56	0.50	0.37
Day 5	0.12	0.56	0.84	1.06	0.52	1.11	0.33	0.37
Average	0.37	0.66	0.62	0.36	0.43	0.57	0.45	0.30
Average±SD	0.47±0.13							



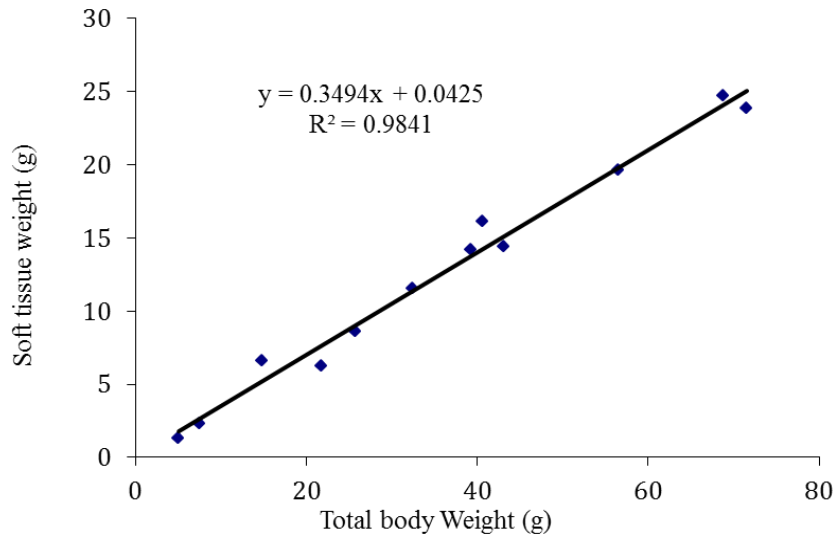
**Figure 2.7.** The clear circle area of biofilm was eaten by opihi

The weight of mass film was calculated in the Table 2.4. The average dry mass per certain area was  $0.013 \text{ g/cm}^2$ . The ratio between the dry weight mass/wet weight was approximately 10 % ( $0.13/1.3$ ; w:w).

**Table 2.4.** Weight of mass film

Replication	Wet weight (g)	Dry weight (g)	Size (cm <sup>2</sup> )	Thickness (mm)	Dry mass g/cm <sup>2</sup>
1	2.10	0.13	12.0	4.00	0.011
2	1.10	0.12	9.60	3.00	0.013
3	0.98	0.11	6.30	3.00	0.017
4	0.74	0.09	7.40	3.00	0.012
Average	1.30	0.13	8.80	3.30	0.013

The relationship between total weight and soft tissue weight is showed in Figure 2.8. A relative equation was  $y = 0.349x + 0.043$  with a coefficient relation  $R^2 = 0.98$ , which is close to 1, suggesting that there is high relatively correlation between total weight and body weight.



**Figure 2.8.** The relationship between the soft body weight and total body weight of opihi

## DISCUSSION

Our purpose was to understand what opihi eats in the wild and what they prefer in their natural eating would be a good starting point to develop an optimum feed palatability factors for them. Examination of stomach content is one practice for the study of diets and food habits of fish and other marine vertebrates (Hyslop 1980). Different studies used various analytical methods to examine the dietary important for fish such as occurrence, numerical, volumetric, gravimetric and subjective methods. No studies have been done to determine dietary important from stomach of opihi. Although previous study reported that opihi feed on benthic crustose coralline algae (Kay and Magruder 1977, Bird 2007), the authors did not attempt to identify benthic diatoms and feeding preferences for opihi were not known. Corpuz (1981) reported that the opihi *C. exarata* grazed on microalgae and young thalli of macroalgae growing on the rocks, but she did not report on the specific stomach contents. Our study is the first report in detail on the stomach contents of opihi. Although our study was qualitative, it went further than previous studies. While much of material we examined was partially digested or amorphous material. However, of what could be identified was diatoms species which were the most frequently seen in the gut contents of examined opihi. In agreement with Hyslop (1980) that the advantages of this frequency of occurrence method provide a crude qualitative picture of the food spectrum.

The weakness of our analysis was only about 30 % of stomach contents could be identified as diatoms. Much of the materials seen were amorphous. Our results prove that diatoms are the most preferred feed for opihi. Our observations were that opihi feed at night. This showed that opihi are a nocturnal feeding species. Therefore, partially eaten foods at night seem to be digested when we collected the animals. Thus, our study confirmed that they are graze on benthic diatoms, bacteria, and whatever else they could find on the rocks. We found about 20 diatom species, which were most frequently seen in the stomach of opihi. These include *Nitzchia*, *Rhizosolenia*, *Melosira*, *Cosinodiscus* and *Navicula* and other.

The biofilm grew spontaneously at a seaside laboratory, where they were naturally recruited from the ocean through a sand filter and exposure to sun light. The biofilm grew fastest in 7 days period on rough plastic sheet as compared to clear and thicker plastic sheets, which took about 14 days for biofilm to cover on surfaces. However, the durability of rough plastic seemed not to be last long as compared to thicker polyethylene. Thus, results suggest that a rough plastic sheet with thicker size should probably be recommended in term of biofilm growth and durability use in practice aquaculture system. Capinpin (2007) reported that plastic plate was good substrate for diatom growth and larval settlement of abalone. Mylar plastic was also tried for larvae settlement of opihi (Corpuz 1983).

The cultured biofilm mat was identified as being dominated by a mixture of benthic diatoms. Similar definition by Nicotri (1977), marine biofilms is the communities of microorganisms containing diatoms, bacteria, and other organisms. Moreover, the organisms recruited were similar to those seen in stomach contents of animals captured from the wild. This consistent suggests that opihi preferred to feed on mostly benthic diatoms. Biofilm was well eaten (0.47%DM/BW/day) by opihi. They survived well. This feed rate will be used as a reference level. Opihi may prefer to feed on benthic diatoms because they are rich in protein and fat. Gordon *et al.* (2006) reported that benthic diatoms *Amphora*, and *Navicula* rich on total lipids (6.5%-14.5% dry weight). Benthic diatoms contain various protein level ranged from 6-52 % (Brown *et al.*, 1997). Thus biofilm is a natural preference diet for opihi and could be used to hold animals alive between feeding trials in the further study. Benthic diatoms could be a larval feed for opihi.

Biofilm represents a dynamic potential chemical cues to most marine invertebrate larvae species. Diatoms such as *Navicula*, *Amphora*, *Nitzchia* and others contain chemical attractant

that induce settlement of abalone larvae (Searcy-Bernal *et al.*, 1992; Gordon *et al.*, 2004; Gapasin and Polohan 2005). Similarly, our preliminary study also found that these benthic diatoms may be attractive for opihi larvae, but more work needed to be done and will be presented in the next chapter. This finding will be shown in further work on developing artificial feed for opihi. It could also serve as an alternating diet for growth out trials (Hua and Ako 2012).

Beside these advantages of biofilm, there are also numerous disadvantages of natural feed. The cost is one issue. It is relatively expensive to operating culture system such as electricity, facilities and labor. For example, our Big Island Abalone Company must dedicate more than 50 % their water to macroalgae culture and water pumping. Moreover, these natural feeds have to be cultured, and re-cultured because our observation showed that they crashed very often in about 45 days, which is unreliable. In addition, travel to the laboratory growing site is a problem in this study. Thus, an artificial diet that can sustain life animals has to be developed and we will describe in the next chapter.

## **CHAPTER 3 . DEVELOPMENT OF ARTIFICIAL DIET FOR OPIHI: FEEDING PREFERENCES**

### **INTRODUCTION**

The focus of this chapter was development of an artificial feed to sustain opihi in captivity. We have learned how to culture the natural feed benthic diatoms in the laboratory which we called biofilm and the natural feeding preferences on biofilm of opihi were determined as 0.47% DM/BW/day. Although the biofilm was effective in sustaining captive opihi, it is a cultured natural feed that would occasionally crash and therefore it is not a sustainable use for aquaculture of opihi. In addition it needed to be cultured near the sea area and going there was inconvenient. The commercial aquaculture of opihi will require the use of an artificial feed. Therefore, we wanted to develop a reliable artificial feed that could completely replace inconvenient natural feed.

The vision was that in order to obtain a commercial feed that produces optimal growth performance, the feed had to have a good palatability and a proper balance of nutrients. We did not investigate nutritional requirements in the present chapter because literature studies on the development of abalone feed were assumed to be good enough. The key feeding preferences of opihi on artificial feed were investigated. The first preliminary feed trials were tried with sea urchin feed to observe whether or not, the artificial feed can be used with opihi and to see how much they eat and as well how long they can survive. This preliminary study showed that animals would tend to starve to death within a week if they did not eat sufficient feed. The highest feed rate was just only 0.12 % DM/BW/day and this was insufficient for maintaining life of opihi. We concluded that the sea urchin feeds were not palatable enough to sustain life of opihi and suggested a need for a modified, formulated diet.

We also discovered that opihi prefer to feed on vertical surfaces, just as they are observed to do in the wild. We thought that a semi-moist diet would be a good choice for animals because it would stick to the side of the feeding container and would be presented to opihi on the side of the aquaria and/or containers. Several trials were then done using fish meal and soy meal as base protein sources with gelatin or agar as binding agents. We found out on hindsight that gelatin and agar were not necessary and not drying feed allowed it to stick to the side of the feeding container. We discovered that several feed ingredients were slightly preferred and some that

were not. Several chemical attractants were tested including betaine, gamma aminobutyric acid (GABA), dimethyl propiothetin (DMPT), but these did not enhance feeding.

An opening window for feed palatability for opihi was biofilm. Adding biofilm into a diet improved feed consumption. The average feeding rate was about 0.17% DM/BW/day, which was increased about two times higher than the feeding rate as compared with control feeds. Biofilm is a natural product. Replacing biofilm with commercial algae preparations was then tried. We found that *Porphyra* preparations doubled feed rates the same as biofilm. We concluded that *Porphyra* could replace biofilm as a feeding stimulant. This alga is the red algal *Porphyra* sp including *P. yezoensis* and *P. tenera*. It sold as Nori or Laver. The opihi did not like other algae such as kelp (*Ascophyllum nodosum*), kombu (*Laminaria japonica*), and fresh ogo (*Gracilaria pacifica*). Animals have been sustained on diets containing *Porphyra* attractants for more than 40 days.

The literature on abalone nutrition was studied. Casein was initially used as the base ingredient to generate standard diets for abalone (Uki *et al.*, 1985, Uki and Watanabe 1992). The hypothesis was that matching the amino acid profile of feed with the amino acid profile of animal tissue was first proposed by Ako and Dominy (1987). It was apparent that they were following that hypothesis in their studies. They used crystalline amino acids in their studies and Coote *et al.* (2000) did a nice job of determining that they did not leach out of diets faster than protein. Numerous investigators have applied these findings using semi-purified diets in which casein was the major protein ingredient supplemented with crystalline amino acids for abalone feed. Subsequently more practical diets were studied with fishmeal, plant meal substitute for casein, with and/or without supplemental crystalline amino acid. The amino acid profiles of their diets mimicked the amino acid profiles of abalone tissue (Mai *et al.*, 1995, Coote *et al.*, 2000, Sales *et al.*, 2003, Cho *et al.*, 2008, Cho 2010). We considered these as milestones in abalone nutrition. However, these studies did not address palatability.

We hypothesized that nutritional factors were similar between abalone and opihi. Feed palatability would be indirectly responsible for growth performance of opihi, nutritional factors being equal and the results of our study will show that there was a relationship between palatability as judged by feed consumption and growth rate of opihi. Cho *et al.* (2008) found that combination diets of fishmeal, soybean meal, with either krill meal, shrimp meal, or green tea meal performed better than other diets even though amino acid profiles of all diets were

matched for abalone feeds (Table 3.1). We hypothesized that some diets performed better than others because they were eaten at higher levels. Feed consumption rates were not measured in these studies. Other factor such as digestibility is also responsible.

**Table 3.1.** Growth performance of abalone fed the different diets (Cho *et al.*, 2008)

Exp. diets	Weight gain (g)
Casein	8.6 <sup>abc</sup>
Fishmeal	8.0 <sup>bc</sup>
Fish/soy/krill	8.8 <sup>ab</sup>
Fish/soy/corn	7.6 <sup>c</sup>
Fish/soy/shrimp meal	9.4 <sup>a</sup>
Fish/soy/green tea	9.1 <sup>ab</sup>
<i>Laminaria japonica</i>	3.3 <sup>d</sup>

Diets share the same superscripts are not statistically significant (Tukey's HSD,  $P>0.05$ ) difference from each other.

In the study that followed Cho (2010) investigated that a combination of soybean meal with either corn gluten meal or silkworm pupae meal incorporated with 5-10% crystalline amino acid. Soybean meal combined with cornmeal or silkworm pupae diets performed well (Table 3.2) and showed differences in growth rates that could not explained by amino acid profile. We hypothesized that the difference in growth rate could due to palatability of diets. As indicated previously, digestibility could also be a factor.

**Table 3.2.** Growth performance of abalone fed the different diets (Cho 2010).

Experimental diet	Weight gain (g)
Fishmeal	5.9 <sup>c</sup>
Soy meal	6.2 <sup>bc</sup>
Poultry	1.9 <sup>d</sup>
Corn meal	1.3 <sup>d</sup>
Silkworm pupae meal	2.0 <sup>d</sup>
Meat and bone meal	1.0 <sup>d</sup>
Soy/corn meal	7.0 <sup>ab</sup>
Soy/silkworm pupae	7.3 <sup>a</sup>
Corn/silkworm pupae	0.9 <sup>d</sup>

Diets share the same superscript are not significantly ( $P > 0.05$ ) different from each other.

In the literature cited above, fish/soy/shrimp (Table 3.1) performed exceptionally well. However, shrimp head meal is of obvious concern and not really advised for Hawaii. The Oceanic Institute no longer uses shrimp meal to avoid disease outbreak as much of it is contaminated with whitespot virus. Another diet of fish/soy/green tea by product also showed good growth rates, but it should be discounted for commercial purposes because green tea meal is not readily available. Similar to Cho (2010), both soy/corn gluten and soy/silkworm diets (Table 3.2) performed well, but soy/silkworm diet should be discounted because silk worm pupae meal is not readily available. Casein diet is also promising but it is too expensive to try. Fish meal and soy meal have high potential use as sole protein sources for opihi feed study. These protein sources could be used singly or in combination with others such as krill or corn meal.

In studies to be described, we will demonstrate that some feeds perform better than others. We speculate that palatability would be responsible for the differences in the growth performance of opihi. Three diets were tested for opihi preferences. The first one was a combination diet of soybean meal, corn meal and fishmeal. The second diet consisted of fish meal, soybean and krill meal. The third diet contained fishmeal only served as control.

Commercial algae *Porphyra* was incorporated into all diets. Two other control diets were a natural feed (biofilm) and an alternating diet of soy/corn/fish with biofilm at 4 day intervals. This feeding method is used for most of commercial abalone farms in the world including the Big Island Abalone Company (Kona, Hawaii). Macroalgae called Dulse (*Palmaria mollis*) has been used as an alternative feed (every 4 days) for commercial abalone culture. Naidoo et al (2006) also reported that abalone *Haliotis midae* grew well on a mixed diet of formulated feed and live algae. In addition, we concentrated on matching the amino acids profiles of feeds with the amino acids profiles of the animal tissue by using fish meal to boost up lysine level instead of adding of crystalline amino acids.

At the end of this chapter we conclude that feeds are close to the best diet that could be formed as a basic diet for grow-out. A diet of fish meal, soybean meal and krill meal produced the best growth (0.44 % body weight/day) and feed intake (0.73% DM/BW/day) for 10 weeks. Similarly, the growth rate for an alternating diet of soybean meal, corn gluten and fishmeal with biofilm was 0.44 % body weight/day and but feed intake was higher at 0.81% DM/BW/day. However, alternative diet was not a good practical method because opihi could be stressed due to transfer between biofilm tank and feeding tank. Therefore, the fish/soy/krill has a potential as basic formulated diet for aquaculture of opihi.

## **MATERIALS AND METHODS**

Preliminary feeding preferences on sea urchin feed. The first feed trials were started with five sea urchin feeds, which were obtained from Addison Lawrence. They all contained the same kelp and alginate binder background, and a mineral package, but differed in their protein component. The basic protein components were fish/squid (6%:6%), the second diet was only fish (12 %) without squid, the third one of 12 % squid without fish, the fourth one was mussel homogenate mix and the fifth one of squid homogenate mix.

Experimental animals. The animals were collected and handled as described in previous chapter. Three to six individuals were tested with each feed. The use of animals was very conservative because we did not want to kill them when they don't eat for a few days at this time in point. For the first trial with sea urchin feed, each diet was run for 30 days. Each individual was weighed and placed into a rectangular aquarium (75 cm x 30 cm x 30 cm; length, width, height). This was repeated until the first treatment was stocked. The selection was then repeated

for each treatment until the last treatment. This selection technique raises the possibility of biased stocking animals and evidence may be seen of this. Two individuals served as a control, they were offered biofilm on rocks instead of extruded feed. An aquarium biofilter system was used (580 L per hour) to simulate water movement. Water quality parameters were temperature, 24-25.5 °C, pH 7.86-8.56, and the dissolved oxygen (D.O) 5.5-6.18 mg/L. We note that these experiments were done before we discovered that lining containers with plastic sheet prevented animal damage and mortality during transfer.

An amount of feed at 1.5-3.0 cm (about 45-200 mg) of sea urchin was fed every day at 17:00. Amount of feed (cm) eaten was judged the next morning. Uneaten feed was siphoned and dried under sunlight for a day and weigh to confirm amount of feed eaten. Feeding data were generally recorded as percentage dry matter (DM) per bodyweight (BW) per day.

Feed ingredients palatability and additional attractants tests. Several gelatin bound diets were tested in this experiment. The first preliminary diet was called fish/soy (Table 3.3). The second diet was a herbivore diet containing soybean meal and corn gluten meal. The attractant betaine at level of 0.2% was also tested and was incorporated with the two diets for this trial. In all diets, corn starch was used to round out the formation to 100 % dry ingredients.

**Table 3.3.** Ingredients composition (% dry matter) of gel diets

Ingredients	Fish/soy	Soy/corn
Fish meal	15.0	-
Soybean meal	10.0	35.0
Corn meal	-	10.0
Spirulina	14.0	14.0
Corn starch	30.0	10.0
Gelatin	18.0	18.0
Alginate	9.00	9.00
CO/MFO*	3.00	3.00
Vitamin mix**	1.00	1.00
Total	100	100
Water	200	200

\*A mixture of corn oil and menhaden oil (1:1, v/v)

\*\* Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute).

This test was conducted with individuals in their own plastic sheet lined rectangular aquaria divided into section. Each trial was run for 5 to 7 days. Tests were terminated if animals did not eat for a few days and risked starving to death. Three squares of feed pieces (1.0 cm<sup>2</sup>/piece; 140 mg mean weight; 32 % dry weight) were fed in the evening. In the next mornings, the % of the eaten squares of feed were judged and converted to dry weight. In some cases, the left over feeds were siphoned and dried in the oven for 5 hr and re-weighed to obtain the weight of uneaten feed. Feed consumption data were also converted to percentage dry matter per bodyweight per day. Animals were left in their containers and animals in the first two treatments were re-used for the third and fourth (betaine) treatments. In this instance the data obtained may be problematic.

Although gelatin was used for abalone diet (Mai *et al.*, 1995), we found that gelatin diets were a hard and inflexible. The formulated diet was then modified by replacement of gelatin with agar. Agar was used as binder (Gorfine 1991). The formulated diets are shown in the Table

3.4. The basic diet containing fish meal and soybean meal was selected because the opihī consumed it at a higher level compared to the soybean and corn basal diet in the second trial. The second diet listed is the same diet with no Spirulina. The third was a biofilm diet in which wet biofilm replaced Spirulina on dry matter basis. The fresh biofilm contained 90% water and water addition at the end was reduced to take into account the water already in the biofilm. The fourth and fifth diets, gamma aminobutyric acid (GABA) was added at ten times the recommended dose (0.45  $\mu\text{M}$ ) and dimethyl propiothetin (DMPT) was added at 3 times higher the recommended dose (recommended dose was 0.1g/kg) respectively.

**Table 3.4.** Ingredients composition (% dry matter) of diets with additional attractive.

Ingredients	Fish/soy/ Spirullina	Fish/soy/ no Spirulina	Biofilm	GABA	DMPT
Fish meal	15.0	15.0	15.0	15.0	15.0
Soybean meal	10.0	10.0	10.0	10.0	10.0
Spirulina	14.0	-	-	-	-
Biofilm <sup>*</sup>	-	-	14.0	-	-
Starch	36.0	50.0	36.0	50.0	50.0
Agar <sup>**</sup>	12.0	12.0	12.0	12.0	12.0
Alginate	9.00	9.00	9.00	9.00	9.00
Corn oil	1.50	1.50	1.50	1.50	1.50
Menhaden oil	1.50	1.50	1.50	1.50	1.50
Mix Vit. <sup>***</sup>	1.00	1.00	1.00	1.00	1.00
GABA	-	-	-	10µl	-
DMPT	-	-	-	-	0.030
Total	100	100	100	100	100
Water	200	200	74.0	200	200

<sup>\*</sup>Fresh biofilm was prepared for at least 10 days in the sun with flowing seawater. It contained 90% water. Thus, amount of wet biofilm added was ten times the amount listed above and additional water was reduced to 74%.

<sup>\*\*</sup> Agar was a commercial agar powder

<sup>\*\*\*</sup> Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute)

GABA, gamma aminobutyric acid, Sigma Chemo.Co.

DMPT, dimethyl propiothetin, Sunheat, China

The third trial was conducted like the second one that after sequential stocking, attempts were made to randomize treatments. Animals were, however, re-used for the last two treatments.

For the fourth trial, feeding stimulant studies were conducted to confirm the preference for diets containing fish meal, soy meal and biofilm and the replacement of biofilm with commercial algae preparations (Table 3.5). The first diet incorporated biofilm into a diet containing fish meal and soy meal. This diet seemed to be most preferred in our third trial. The next also

contained biofilm but also contained a higher fish meal level to evaluate whether the presence of higher fishmeal levels improves feed consumption. The third diet was a control with high fish meal but without fresh biofilm and acted as a no-biofilm control. The last one was in which biofilm was replaced by dried, commercially available alga “nori” *Porphyra tenera* or *P. yezoensis* (Nishimoto Trading Co. Ltd., Korea). This trial was continued with substitution of three other commercial algae preparations for biofilm; Norwegian kelp (*Ascophyllum nodosum*), commercial brown algal kombu (*Laminaria japonica*), and fresh ogo (*Gracilaria pacifica*) for biofilm.

**Table 3.5.** Ingredients composition (% dry matter) for final taste test diet.

Ingredients	Fish/soy/ Biofilm	High fishmeal/ biofilm	High fishmeal/ no biofilm	High fishmeal/ Porphyra
Fish meal	15.0	30.0	30.0	30.0
Soybean meal	10.0	10.0	10.0	10.0
Biofilm *	14.0	14.0	-	-
Porphyra **	-	-	-	14.0
Corn Starch	35.6	20.6	34.6	20.6
Agar ***	12.0	12.0	12.0	12.0
Alginate	9.00	9.00	9.00	9.00
Corn oil	1.50	1.50	1.50	1.50
Menhaden oil	1.50	1.50	1.50	1.50
Mixed Vit. ****	1.00	1.00	1.00	1.00
Cholesterol	0.40	0.40	0.40	0.40
Total	100	100	100	100
Water	74.0	74.0	200	200

\* Fresh biofilm was prepared for at least 10 days in the sun with flowing seawater. It contained 90% water. Thus, amount of wet biofilm added was ten times the amount listed above and additional water was reduced to 74%.

\*\* This is commercial seasoned seaweed known as nori or the red algae *tenera* or *yezoensis*. (Nishimoto Trading Co., Ltd, Korea)

\*\*\* Agar was a commercial agar powder (Vietnam)

\*\*\*\* Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute).

Growth performance of opihi on artificial feed. For the fifth set of feeding trials, the three diets shown in Table 3.6 were tested. Diet 1 contained soybean and corn gluten meal with fishmeal, the presence of fishmeal was used to increase the lysine level instead of using crystalline amino acid. This diet also contained *Porphyra* for superior palatability and was considered a herbivore control diet. The second diet was a combination diet of fishmeal, soybean meal, krill meal and *Porphyra*. Ingredients were chosen in part based on Cho *et al.* (2008). The third diet was also a control diet, in which fishmeal was used as a sole protein and *Porphyra* was used as an attractant. The fourth treatment alternated feeding of soy/corn/fish diet described previously and cultured biofilm once every 4 days. This diet was chosen because most commercial abalone nurseries worldwide rely on macroalgae feeds as a supplementary food source for post larvae and juveniles (Daume *et al.*, 2000). In the last treatment, the animals were fed on cultured biofilm. It also served as a different kind of control. Biofilm was chosen as control because in our previous study the animals fed well on the biofilm as their natural food which contains most of benthic diatoms were identified by stomach content analysis. Feed consumption on biofilm was determined as described previous chapter.

**Table 3.6.** Ingredients (% dry matter) of the diets.

Ingredients	Soy/corn/fish/	Fish/soy/krill/	Fishmeal/
	porphyra	porphyra	porphyra
Fishmeal	20.0	25.0	35.0
Soybean meal	29.0	20.0	-
Corn gluten	18.0	-	-
Krill meal	-	13.0	-
Porphyra *	14.0	14.0	14.0
Wheat flour	11.3	20.3	43.3
Alginate	5.00	5.00	5.00
Corn oil	1.00	1.00	1.00
Menhaden oil	0.30	0.30	0.30
Vitamin mix **	1.00	1.00	1.00
Cholesterol	0.40	0.40	0.40
Total	100	100	100
Water	133	133	133
Proximate analysis			
Protein	45.0	40.0	32.0
Lipid	8.90	11.0	7.60
Carbohydrate	24.0	29.0	42.0

\*The commercial algae *Porphyra tenera* or *yezoensis*;

Nishimoto Trading Co. Ltd., Korea

\*\* Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute).

Experimental animals. In the last trial (above), six to ten animals (2.5-4.0 cm shell length) were tested for each diet. They were selected from the holding aquaria and placed into the colander (20 cm diameter), one animal per colander, two colander per an aquarium with a biofilter system (580 L per hour) to simulate water movement. Treatments were assigned somewhat randomly and animals were re-used for some of the later diets. In some cases two animals in the same aquarium was used. For the biofilm diet, the test was conducted at HIMB

and three animals were placed into different locations in biofilm aquarium, three aquaria were used.

**Dietary preparation.** Dietary preparation was modified from the method of Cho (2010). A typically 3 g of dry ingredients and a volume of 6 mL of water was added into a beaker (25 mL) containing corn starch, gelatin/agar and alginate. The beaker was then placed into the boiled water beaker (250 mL) on a hot plate and stirred while being heated. The gelatinization occurred when the water temperature increased in a few minutes, the solution became more and more viscous and turned into a thick gel. Other ingredients were then mixed thoroughly with the gelatinized solution, thereafter the mixed (paste) was heated in boiled water bath again for about 2 minutes. The paste was shaped into sheets about 1.0 mm thickness, and then cut into 1.2 cm<sup>2</sup>/pieces and dried naturally in laboratory conditions for about 1-2 hr. The pieces were then sealed in a plastic sample bag and stored at -20 °C until use. The feeds contained 32 % dry matter as determined by weighing wet, drying, and re-weighing.

Amino acids profiled of these diets were analyzed at Oceanic Institute according to the method of Ju *et al.* (2008).

**Data analysis.** All feed consumption of animals was calculated and averaged by Excel. One way ANOVA follows by Tukey test was used to compare mean feed consumption, weight gain and specific growth rate between the trials. Growth performance of opihi was expressed as specific growth rate (SGR):  $SGR = \{ \ln W(f) - \ln W(i) / T \} \times 100$ . Where W(i) = initial body weight, W (f) = final body weight and T = time in days.

**Water stability test.** Gelatin and agar were used initially as binders. Experimental trials were undertaken with removing agar from the diet. Table 3.7 shows the formulated feeds were made by different agar levels. This study was tested to access whether or not the presence of agar would be necessary for feed stability.

**Table 3.7.** The composition of diets with various agar levels.

Ingredient	0% agar	3% agar	6% agar	9% agar	12% agar
Fishmeal	35.0	35.0	35.0	35.0	35.0
Porphyra	14.0	14.0	14.0	14.0	14.0
Corn starch	43.3	40.3	37.3	34.3	31.3
Agar	0.00	3.00	6.00	9.00	12.0
Alginate	5.00	5.00	5.00	5.00	5.00
Corn oil	1.00	1.00	1.00	1.00	1.00
Menhaden oil	0.30	0.30	0.30	0.30	0.30
Vit mix	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.40	0.40	0.40	0.40	0.40
Total	100	100	100	100	100
Water	133	133	133	133	133

Feed stability with different alginate levels was tested as shown in Table 3.8.

**Table 3.8.** The composition of proposed diets with various alginate levels.

Ingredient (%)	0%	3%	6%	9%
Fishmeal	35.0	35.0	35.0	35.0
Porphyra	14.0	14.0	14.0	14.0
Corn starch	36.3	33.3	30.3	27.3
Agar	12.0	12.0	12.0	12.0
Alginate	0.00	3.00	6.00	9.00
Corn oil	1.00	1.00	1.00	1.00
Menhaden oil	0.30	0.30	0.30	0.30
Vit mix	1.00	1.00	1.00	1.00
Cholesterol	0.40	0.40	0.40	0.40
Total	100	100	100	100
Water	133	133	133	133

Feed stability tests were done with four replicate pieces (1.0 cm<sup>2</sup>/piece). Tests were conducted in about 180 mL of seawater in 200 mL polystyrene cups at room temperature (approximately 23°C). Stability of feed was assessed as the equation: Water absorbed (%) = {(FW – IW)/IW} x 100. Where IW is initial weight of feed, and FW is the final weight of feed of the same diet after being immersed in water for a certain period of time at 1, 3, 6, and 12 hr.

## RESULTS

Preliminary test with sea urchin feed. The trial using sea urchin feeds showed that the diets containing fish and squid seemed to be preferred, whereas feeds containing homogenates of mussel and squid were not as eaten well (Table 3.9). The best feeding rate was 0.12 %DM/BW/day for fish/squid and the same 0.02 % DM/BW/day for both mussel and squid homogenate although values may have been distorted by stocking bias. Stocking bias would put the strongest, most robust, and best performing animals in the first stocked tanks and weaker and weaker animals in other tanks.

The animals did not eat in the morning feeding time. In this experiment, the animals also fed infrequently, an average of once every 3 nights. A high mortality (35 %) occurred in two days after beginning of the trial may have been due to handling while subsequent mortalities (17 %) may have been due to insufficient feeding for a few days (5-6 days) after starting. Dead animals were eliminated from the feed consumption calculations. This trial must be considered preliminary because of low survival.

**Table 3.9.** Feed consumption (%DM/BW/day) of opihi fed sea urchin feeds.

Feed style	Animals	Feed consumption	Last day alive
6% fish 6% squid	3	0.12±0.05	30, 3, 6
12% fish 0% squid	3	0.05±0.06	3, 30, 30
0% fish 12 % squid	3	0.09	30, 2, 1
Mussel Homogenate	3	0.02±0.00	30, 30, 5
Squid homogenate	3	0.02	30, 2, 2
Control (Biofilm)	2	<i>Ad libitum</i> feeding	30, 16

Examination showed that animals that have eaten have a lighter muscle color than animals that have not eaten (Fig. 3.1). Animals that have not fed have a black muscle color usually indicating stress in marine animals.



**Figure 3.1.** A difference between an opihī that eaten feed (left) and one that ate little or no feed (right).

Feed ingredients palatability and additional attractants test. Fish/soy and soy/corn diets were tried and betaine was tested as an attractant (Table 3.10). Statistical analysis showed that there is a significant difference ( $P = 0.009$ ) in feed consumption of opihī in between different diets. The diets containing fishmeal and soy meal was eaten at the highest rate but did not differ significantly compared to a herbivore diet with soy and corn meal. The attractant betaine did not help to improve feed consumption relative to the basic fish/soy diet. Animals fed relative frequently with the best two diets having an average of 2.7 feedings among the three animals eating the first mentioned diet over 5 days. Feeding was less frequent in diets containing betaine, the average feeding was 1 feeding out of 5 days testing of three animals. The average feeding rate was very low at 0.03%DM/BW/day for fish/soy/betaine and 0.01%DM/BW/day for soy/corn/betaine. This unusual feeding behavior could be due to the fact that animals were recycled and were becoming less and less fit as time progressed. All the animals survived the 10 day (total) duration of the trial.

**Table 3.10.** Feed consumption of individual animal in each diet (n=3).

Day	Feed consumption (% dry matter/body weight/day)											
	Fish/soy			Soy/corn			Fish/soy/betaine			Soy/corn/betaine		
1	0.41	0.25	0.00	0.25	0.25	0.41	0.00	0.45	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.25	0.00	0.03	0.00	0.00	0.02	0.02	0.02
3	0.27	0.15	0.17	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05
4	0.41	0.38	0.34	0.00	0.12	0.12	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.0.	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aver. ±	0.22	0.16	0.1	0.06	0.12	0.11	0.01	0.09	0.00	0.004	0.004	0.02
SD	0.16±0.06 <sup>a</sup>			0.10±0.03 <sup>ab</sup>			0.03±0.05 <sup>b</sup>			0.01±0.01 <sup>b</sup>		

Diets share the same superscripts are not statistically significant (Tukey's HSD,  $P > 0.05$ ) difference in mean feed consumption from each other diets.

Additional attractants were tested. The preference among Spirulina, no Spirulina, biofilm, attractant GABA, and DMPT diets were tested (Table 3.11). Feed rates varied among the animals led to a high and variable standard deviation of feed consumption among the animals in various diets. The diet incorporating biofilm was most and significantly ( $P < 0.05$ ) preferred and the animal fed relatively frequently (53 % of the nights). Diets containing no Spirulina and Spirulina without biofilm, GABA, DMPT were not as well eaten and the feeding was not frequently at a rate of about 30 % of the nights for all diets. This trial suggested that biofilm could be a key for a chemical cues enhancing feeding. In this trial, the influence of biofilm was so profound that it overcame data scatter due to stocking bias or recycling of animals.

**Table 3.11.** Feed consumption of individual animals fed various diets incorporating attractant (n=3).

Day	Feed consumption (%dry matter/body weight/day)														
	Fish/soy/Spirulina			Fish/soy/no Spirulina			Fish/soy/Biofilm			Fish/soy/GABA			Fish/soy/DMPT		
1	0.00	0.13	0.00	0.03	0.00	0.00	0.21	0.30	0.32	0.25	0.00	0.05	0.04	0.00	0.00
2	0.02	0.02	0.03	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.10	0.13	0.12	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00	0.60	0.00	0.20	0.32	0.00	0.00	0.00	0.00	0.20
4	0.15	0.00	0.00	0.10	0.00	0.05	0.00	0.45	0.00	0.00	0.25	0.00	-	-	-
5	0.00	0.00	0.00	0.00	0.14	0.00	0.28	0.00	0.12	0.00	0.00	0.06	-	-	-
Aver.	0.03	0.03	0.01	0.03	0.03	0.01	0.22	0.15	0.13	0.11	0.08	0.05	0.05	0.0	0.07
Aver. ±SD	0.02±0.01 <sup>a</sup>			0.02±0.01 <sup>a</sup>			0.17±0.19 <sup>b</sup>			0.08± 0.11 <sup>a</sup>			0.04±0.07 <sup>a</sup>		

Diets share the same superscripts are not statistically significant (Tukey's HSD,  $P>0.05$ ) difference in mean feed consumption from each other diets.

In the follow up tests, fishmeal and fishmeal at twice amount the baseline level was tested with a different set of attractants (Table 3.12). The results showed that increase fish meal did not improve feed consumption rate. Removing biofilm drastically and significantly decreased feeding which confirms the previous results. In the attempt to find to a commercial replacement for biofilm the study showed that the additional of commercial algae *Porphyra* was the most preferable among commercially available algae as an attractant and was as attractive as biofilm. One-way ANOVA analysis revealed that the opihī showed a significant preference ( $P < 0.0001$ ) when offered different algae incorporation with diet. Statistical analysis by Tukey test revealed that the opihī ate significantly more diets with biofilm or *Porphyra* than the other diets. Feed consumption was at the same level for both diets. These data suggest that the commercial algae *Porphyra* sp completely replaced biofilm.

**Table 3.12.** Confirmatory trials for feed ingredient preferences and substitution biofilm with commercial algae.

Diet	# animals tested	Average days eating/ days tested	%DM/BW/day
Fish/soy/biofilm	6	3/6	0.08±0.03 <sup>a</sup>
High fish/soy/biofilm	6	2.5/6	0.07±0.01 <sup>a</sup>
High fish/soy/no biofilm	6	0.5/4	0.02±0.02 <sup>b</sup>
High fish/soy/ <i>Porphyra</i> *	3	3/6	0.09±0.04 <sup>a</sup>
High fish/soy/kelp**	4	0.75/4	0.04±0.03 <sup>ab</sup>
High fish/soy/Kombu***	4	0.5/4	0.03±0.03 <sup>b</sup>
High fish/soy/ogo****	4	0.5/4	0.03±0.03 <sup>b</sup>

Note: Values share different superscript letters are significant differently ( $P < 0.05$ ), within the column.

\*This is commercial seasoned seaweed known as nori or the red algae *Porphyra tenera* or *yezoensis*. Nishimoto Trading Co. Ltd., Korea

\*\* Norwegian kelp *Ascophyllum nodosum*, from Oceanic Institute

\*\*\*This is commercial seaweed known as kombu or the brown algae *Laminaria japonica*, Nishimoto Trading Co. Ltd., Japan.

\*\*\*\* Fresh ogo known as red algae *Gracilaria pacifica*, super market Donquijote, Honolulu

Growth performance of opihi during final trials. Initial, final weight, the growth rates, feed consumption and survival of the animal for 10 weeks are shown in Table 3.13. The weight gain of animals was normalized for each individual. Animals gained weight in all diets, with the highest achieved weight gain of 28 % and 33 % body weight in the 10 week period when opihi that were fed with fish/soy/krill and alternating diet of soy/corn/fish with biofilm diets, respectively. There was a significant difference ( $P < 0.05$ ) between the initial and final weight gain of opihi that fed by fish/soy/krill and alternating diet. The biofilm diet showed surprisingly poor growth. Statistical analysis by one way-ANOVA indicated that there was significantly different ( $P < 0.0001$ ) effects from experimental diets, following by Tukey's test showed that there was significant difference ( $P < 0.05$ ) in the weight gain of opihi that were fed fish/soy/krill and alternating diets, compared with other diets. Similarly, the specific growth rates increased for opihi fed diets fish/soy/krill and alternating were significantly higher compared to those fed with other diets. However, alternating feeding between artificial feed and biofilm was problem because when placed in a container the tested animals did not eat for two days. They then fed for two days. At this point they would be transferred to another tank for feed change and the feeding again stopped for 2 days before returning to normal feeding. The meaning of the data is questionable due to non-feeding day.

Fish/soy/krill produced the highest feeding rate ( $0.73 \pm 0.53$  %DM/BW/day) among the formulated diets, whereas fishmeal diet was the lowest in feed consumption (Table 3.13). Statistical analysis revealed that there was a significant difference ( $P < 0.05$ ) in feed consumption among the formulated diets. Diet of soy/corn/fish was consumed moderately. For the alternating diet, the artificial diet was eaten at a higher rate than biofilm. The feed consumption of the control biofilm was estimated to be the highest at 0.81 %DM/BW/day. Feeding frequently in all diets was about 45 % of the total number of the feeding day (approximately one feeding for every 2 days).

**Table 3.13.** Total body weights (g), SGR (% day<sup>-1</sup>), shell length (cm), feed consumption (%DM/BW/day), survival (%) of opihi fed by different diets for 10 weeks.

	Soy/corn/fish /porphyra	Fish/soy/krill /porphyra	Fish/ porphyra	Alternating diet*	Biofilm
Initial weight	8.89±0.10	6.51±3.40	8.70±1.70	4.76±2.18	5.04±0.62
Final weight (g)	9.65±0.65	8.31±3.24	10.69±0.35	6.33±2.38	5.42±0.65
Weight gain (g)	0.76±0.45 <sup>a</sup>	1.80±1.38 <sup>b</sup>	0.78±0.88 <sup>a</sup>	1.58±0.62 <sup>b</sup>	0.38±0.41 <sup>a</sup>
SGR	0.12±0.07 <sup>a</sup>	0.44±0.37 <sup>b</sup>	0.11±0.13 <sup>a</sup>	0.44±0.19 <sup>b</sup>	0.10±0.11 <sup>a</sup>
Initial shell length (cm)	3.96±0.38	3.26±0.70	3.48±0.20	2.91±0.91	2.84±0.25
Final shell length (cm)	4.30±0.03	3.28±0.76	3.59±0.12	3.29±0.35	3.15±0.11
Feed consump. (%DM/BW/day)	0.18±0.07 <sup>a</sup>	0.73±0.53 <sup>b</sup>	0.15±0.03 <sup>a</sup>	**0.51±0.29 <sup>ab</sup>	***0.81±0.54
Survival (%)	67	67	67	71	50

Note: In the same row, values sharing the same superscript letter are not significantly different ( $P > 0.05$ )

\*alternating feeding of soy/corn/fish and biofilm for every 4 days

\*\*sum of formulated feed (0.32±0.19 %DM/BW/day) and biofilm (0.19±0.10 %DM/BW/day);

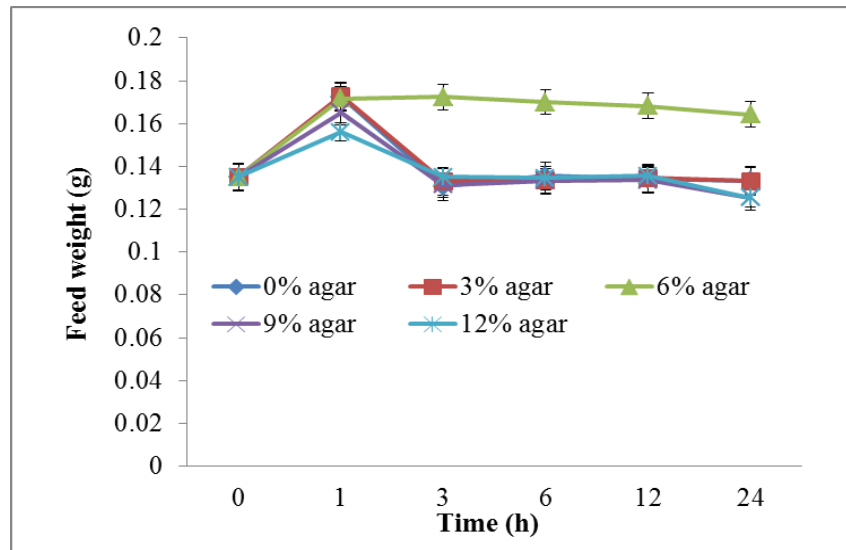
\*\*\*the data was suggested not to use for comparing with other diets as due to overlapping feeding.

The A/E ratio (each essential amino acid/total essential amino acids) x 1000) amino acid profiles showed that most of the essential amino acids of the diets were identical and/or close to amino acid profiles of opihi tissue except for Arg and Lys (Table 3.14). Some of the EAA were low in one diet but higher in others such as Leu was high in soy/corn/fish but lower in fish/soy/corn and fish diets, but Lys was high in soy/corn/fish diet but lower in other diets.

**Table 3.14.** The A/E ratio amino acids profiles of grow out diets and opihi tissue.

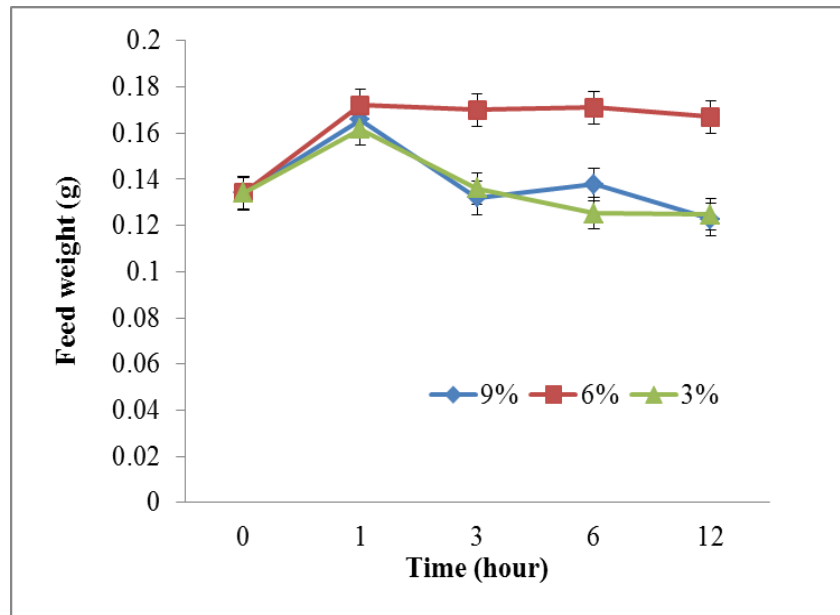
No.	Essential AA	A/E ratio [(each EAA/Total EAA) x 1000]			
		Soy/corn/fish	Fish/soy/krill	Fishmeal	Opihi tissue
1	Arg	110	132	120	160
2	His	42.3	40.8	40.2	24.5
3	Ile	72.5	61.4	60.2	83.5
4	Leu	169	120	114	170
5	Lys	103	122	116	168
6	Met/cys	81.2	90.2	122	80.1
7	Phe/tyr	216	222	208	129
8	Thr	106	104	109	100
9	Val	90.0	108	110	84.1

The “soft” diets were generated in response to the vertical surfaces feeding behavior of opihi. A question was raised on this feed type whether gelatin or agar was required. The results showed that gelatin was not suitable as it appeared to hinder feeding. The stability test showed that agar was not necessary. Alginate was all the binder that the feed needed. Figure 3.2 showed that diets containing different agar levels (0-12 %) and constant alginate level (5 %) remained to stable for a long period of time while immersed in seawater. All the diets absorbed water for the first hour then remained and stable for more than 24 hr. After 12 hr, all the feeds seemed to get softer and was starting to break a little bit when water was agitated.



**Figure 3.2.** Feed weight of various agar levels versus time immersing in seawater, n=3.

Various alginate levels in diet were also tested. The feeds disintegrated immediately when immersed in the seawater for diet contained no alginate. Performance of the other diets containing various alginate levels are shown in the Figure 3.3. The Figure 3.3 shows that water was absorbed for the first hour then the weight was stable for the next hours. Among those diets, the diet containing 3 % alginate started to break down into pieces in the water after 3hr. It was very brittle. It was unclear why the 9 % alginate diet was not stable. Diet contained 6 % alginate remained stable for the full 12 hr in water column.



**Figure 3.3.** Feed weight of various alginate levels versus immersing in seawater by the time, n=3.

Water stability tests were also conducted for different carbohydrate levels. The results will be presented in the next chapter.

## DISCUSSION

Our goal was to develop an artificial feed for aquaculture of the opihi. In the previous chapter we found that biofilm, a cultured natural feed composed of benthic diatoms was eaten well by opihi. It supported survival. However, it is not reliable as natural feeds sometimes crash and are expensive of labor. For example we observed that Big Island abalone used more than 50% of their tanks space to grow macroalgae. Studies on artificial diets for abalone to supplement or replace macroalgae in abalone aquaculture have been intense studied during the last decade. Artificial diets overcome problems with availability, seasonality and die off restrictions and made commercial aquaculture abalone feasible (Uki and Watanabe, 1989). Artificial feeds were also able to meet nutritional requirements and are inexpensively handled and stored.

Sea urchin feeds were not palatable enough to sustain opihi life. Quantitative results are questionable due to stocking bias and handling mortalities during the tests. However, the tests taught us some things. They taught us that animals would tend to starve to death if they did not

eat or ate too little. This suggested that future feed trial should be terminated if the animals do not eat sufficient feed for 5-7 days period. It is noted that this experiment was commenced before the plastic tank liner method was discovered and several mortalities at day 1 and/or day 2 could be due to handling injuries. The feeding rates on sea urchin feeds were much lower than the 0.47%DM/BW/day for biofilm. This trial was also repeated with the three of marine protein of fish/squid, fish, and squid diets with more animals, but the feeding rate were low in all diets (0.02% DM/BW/day). We also learned that the animals did not eat in the morning feeding period. This may be the same as nocturnal behavior also seen in abalone (Uki 1981; Barkai and Griffiths 1987). It is likely that the opihi respond to light in a similarly negative way and are nocturnal. When it is dark, opihi begin searching around the aquarium. When they bump into something, they “feel” it with their tentacles. Opihi preferred to stay on the vertical site of the container, just as they are observed to do on vertical surfaces in the wild. This suggested that a semi-moist feed with gelatin binder could be used at this point in time because it could be placed on the vertical side of the aquaria or colanders. Semi-moist feeds had been applied in most of abalone feed studies (Uki and Watanabe 1986; Mai *et al.*, 1995; Cho *et al.*, 2008; Cho 2010) and gelatin was used as binder (Mai *et al.*, 1995; Knauer *et al.*, 1993). Gelatin and agar were used and would probably be good initial choices if binders were not known. Eventually we proved that both gelatin and agar were not necessary because alginate served as a good enough binder and not drying the feed allowed us to have a semi-moist feed.

Results of the second trial showed that fish meal and soy meal form a baseline diet. The diet containing fish meal and soy meal tended to be eaten in higher amounts (0.16%DM/BW/day) than soymeal and corn meal diet (0.10%DM/BW/day) but not significantly so. Eating rates may be slightly high due to the animals being fresher from the collection. However, betaine added to the diets did not improve feeding rate. Feeding rate was only 0.01-0.03% DM/BW/day and the average feedings frequency was only about 20 % of the feeding nights. This feed rate may be artificially low as animals used had been recycled and may not have been in optimal physical health. The expectation is that feeds containing non-attractant substances such as betaine would have been eaten at the same rates as controls. Instead betaine-containing feeds behaved as if betaine were a repellent. Betaine is an attractant for shrimp and fish, addition of betaine (5g/kg) in a diet produced the highest weigh gain and high feed consumption in prawn *Macrobrachium rosenbergii* (Felix and Sudharsan 2004). In another

study, there was no difference in feed intake among the diets with betaine level from 0.5-2.5g/kg for tilapia (Luo *et al.*, 2010). Nevertheless, our data suggested that fish/soy was liked and could be used as base diet for further trials and attractant betaine did not improve feed rate for opihi. This could probably due to different chemosensory preferences of the opihi.

Biofilm was a key to feed attraction. The incorporating biofilm with fish/soy diet doubled the eating rate compared with controls. It was eaten at the rate of 0.17%DM/BW/day and higher significantly as compared to other attractants and the frequency of feeding was 53 %. Perhaps, biofilm contains chemical cues that act as an attractant. Similar to sea urchin *Tripneus gratilla*, an additional 5 % of natural favorite feed of macroalgae (*Eklonia radita*) produced a better feed consumption compared to diets without algae (Dworjanyn *et al.*, 2007). Enhancement of feeding rate of biofilm supported our previous study on opihi biofilm preferences.

Spirulina did not help to enhance feed consumption or raise the feeding frequency for opihi. The feeding rate was only 0.02%DM/BW/day and feeding frequency was about 20 % of the nights. Like betaine, Spirulina acted like a repellent and there is no explanation. Unlike abalone, Bautista-Terual *et al.* (2003) reported that the present of Spirulina in abalone's diet improved feed intake. Similarly, other potential feeding stimulants such as, GABA, and DMPT did not increase feeding rate and feeding was not frequently (30%) compared to the diet containing Porphyra. GABA is a bioactive molecule widely known for its function as a neurotransmitter which also elicited postlarval *H. asinina* settlement and metamorphosis (Gapasin and Polohan 2004) at a concentration of 0.45-0.5 mM. DMPT has also been used as feed attractants shrimp. Mengqing *et al.* (2002) reported the presence of DMPT (585 mg/kg) in prawn diet improved the feed conversion ratio. Our study suggests that different species may have different feed attractant preferences.

Higher fish meal was not a key to feed palatability. Increasing fishmeal (doubling level) did not increase feed rate. Feed consumption (0.07 %DM/BW/day) remained the same feed rate as diet of lower fishmeal diet (0.08%DM/BW/day). Although, it has been known that fishmeal could improve feed palatability for fish and other animals (Uki and Watanabe, 1986).

A breakthrough was biofilm replacement with *Porphyra* as a feed attractant. Among the palatable dietary ingredients tested in a preliminary way above, fish meal and soybean meal as well as feeds incorporating biofilm were preferred. However, the biofilm diets are a problem

typical of other live ingredients. This is the reason we searched for a commercial substitute. This commercial product was a *Porphyra* preparation that is used among other things to make sushi and is called nori in that context. Feed consumption for *Porphyra* diet (0.09 %DM/BW/day) was similar to diets with biofilm (0.08 %DM/BW/day) in this trial. The diet which incorporated *Porphyra* had the animals feeding frequently and well enough that one animal survived more than 40 days on this artificial diet. The animal grew in shell and width and length after 6 weeks eating the diet incorporated with *Porphyra*. This reveals that *Porphyra* diet could be used to maintain animals in a certain time before and during feed trial without supplying natural feed. Addition of algae into artificial feed also increased feed intake in abalone diet. Uki *et al.* (1985) found that the additional 3 % of *Laminaria japonica* into test diet would improve feed intake in abalone *H. discus hannai*. Another study by Gomez-Montes *et al.* (2003), they used kelp meal as filler, not for feed enhancement purposes in abalone feed. The authors found that there was a significant difference in feed consumption with dietary kelp levels from 9 % to 17 %. A lower significant difference in feed consumption of opihi that were fed with diets containing ogo and kombu compared to *Porphyra* diet indicates that these algae did not seem to work as attractants.

The last feed trial can serve as a summary and a capstone. The non-*Porphyra* or biofilm diets form the baseline. They are eaten at 0.02-0.16%DM/BW/day. The wide range reflects the challenges with animals in this work. They have not growth rates associated with them because they are not eaten at high enough rates to sustain life. *Porphyra* or biofilm containing diets are eaten at higher rates at between 0.07-0.18%DM/BW/day. They form the next step up in palatability and they sustain growth rates of about 0.76-0.78 g/animal/10 wk trial or 0.11-0.12 g/day specific growth rate. The highest step combines fish meal/soy meal with krill meal and includes *Porphyra*. It is eaten at the highest rates of 0.73%DM/BW/day which is about 7-fold higher than the lower level and sustains the highest growth rates of 1.8 g/animal/10 weeks and a specific growth rate of 0.44 %/day. It is eaten in significantly higher amounts than diets not containing krill meal and supports significantly higher growth rates. Harada *et al.* (1985) reported that the abalone were most attracted by certain proteins, or amino acids, non-volatile nitrogens.

In the last trial the alternating diet is interesting. Our final trial has demonstrated that feed palatability plays an important role in growth performance of opihi. It is eaten at moderate rates,

0.51%DM/BW/day in spite of the fact that animals do not feed for about two days after switch diets from artificial feed to biofilm or vice versa. It supports good growth rate in spite of this 1.58 g/10 weeks or 0.44 g/day specific growth rate. It is second only to the fish/soy/krill/*Porphyra* diet in terms of increases in size for the 10 week trial, 28 % versus 33 % for the latter. The high growth in spite of lower feeding might mean that the artificial feed/biofilm diet may contain an unknown growth factor in it. It would probably have to be modified for opihi if used because it requires moving animals from container to container every four days and the opihi would eat nothing for two days when moved. It also comes with the disadvantage of being a natural product that must be grown. The abalone *Haliotis midae* grows well in mixed diet of formulated feed and macroalgae and do the abalone at the Big Island Abalone Company. Naidoo *et al.*, 2006 feed dried kelp alone.

Surprisingly, biofilm diet was eaten well (0.81%DM/BW/day) but performed poorly in growth rate and survival. This is probably due to an inferiority in amino acid profile. Cho *et al.* (2008) reported that a poor in essential amino acid profiles of algal *Laminaria* produced a poor growth rate in abalone.

We are concerned about the stability of artificial feed for opihi because they are slow aquatic feeders. We examined feed stability using gelatin (informally), agar and alginate, and alginate alone. The alginate alone diet was water stable and therefore agar is not necessary for binding. The diet containing 6 % alginate had very good 12 hr stability. This indicated that 6% of alginate would probably be a good binder for opihi feed. Fleming *et al.* (1996) reported that most of abalone diets used alginate as the main binding agent, at the level ranging from 0.8 to 20 %. Our failed studies were similar to study by Knauer *et al.* (1993) who found that the feed bound with 2 % alginate was poorly stable, with about 50 % of dry weight lost after only 6 hr.

## CHAPTER 4 . THE EFFECT OF DIETARY PROTEIN LEVEL ON GROWTH PERFORMANCE OF OPIHI

### INTRODUCTION

The purpose of this chapter was to determine the protein requirement of opihi. A series of systematic experiments were done to investigate and develop an optimal feed palatability which would sustain life and support a good growth rate for opihi. This artificial diet was without live feed ingredients and associated problems. This was reported in the previous chapter. A proper balance of nutrients is obviously necessary in our further steps of the development of opihi feed beside the palatability. Protein is sometimes considering as the first priority macronutrient to be examined in nutritional requirement studies because it is a base component for muscle growth and is the most expensive feed ingredient. The effect of different dietary protein levels on growth performance of opihi was examined in this chapter.

No studies have been done previously on protein requirements of opihi, while there have been studies on the protein requirements of abalone. It is obvious that the hypothesis of matching the amino acids profile of diets with the amino acids profile of animals tissue proposed by Ako and Dominy (1987) was widely used in abalone feed studies. Mai *et al.* (1995) used a mixture of casein and gelatin at a ratio of 4.34:1 to manipulate the increasing in protein level from 10 % to 50 %. Various dextrin levels were used to fill up as the protein increase. This would lead to different amount of carbohydrate levels in these diets. All diets were supplemented with free amino acids (Arg, Thr and Met) to match the amino acids profile of the abalone. The leaching of supplemented crystalline amino acids was studied by Coote *et al.* (2000) and leaching refers to the loss of nutrients from feed due to dissolving in water. They reported that there was a large leaching of supplemented crystalline amino acids (about 44 % to 54 %) and leaching of protein bound amino acids (28 % to 40 %) in 24 hr. The leaching rate was similar for both additional free amino acids and protein-bound amino acid, suggesting that leaching was not an overwhelming issue that distorts the data.

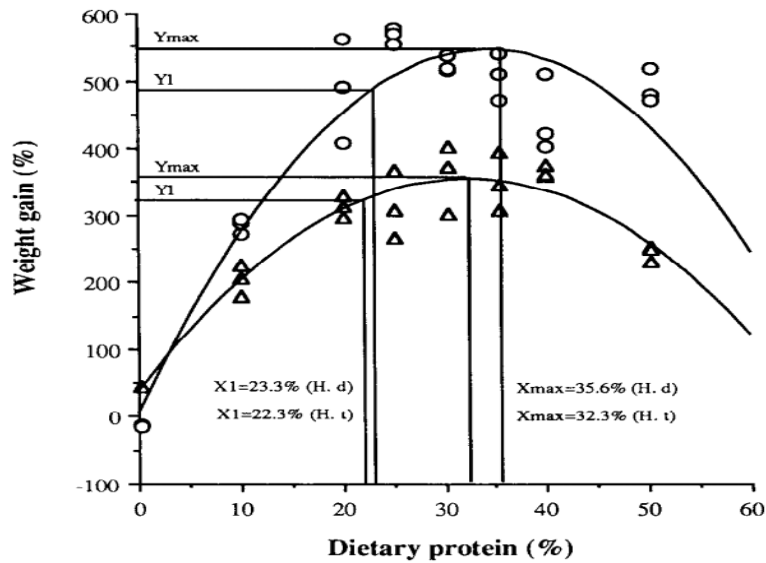
The juvenile abalone were fed with various dietary protein levels ranging from 0.28 % to 50 %. Different statistical methods were used to evaluate the optimum protein level for growth of the two species of abalone. One way ANOVA analysis followed Tukey' test revealed that there were no significant differences in weight gain of opihi that were fed with dietary protein

levels from 25 % to 40 % and 20 % to 50 % for *Haliotis tuberculata* and *H. discus hannai*, respectively (Table 4.1). Second-order polynomial analysis demonstrated that about 35 % dietary protein produced maximum growth in term of weight gain for *H. discus hannai* and 32 % for *H. tuberculata* (Fig. 4.1). These are reported in Table 4.6. Overall these results suggested that a 25 % to 40 % protein level in diet was probably the protein level that produces good growth in abalone. The broad protein requirement probably means that the lowest significant protein level satisfies the animal's need and higher levels are not harmful. The optimal level as determined by polynomial analysis has no significance and is therefore only a convenient way of stating the non-significant protein requirement. Protein requirements should be tested in opihi.

**Table 4.1.** Effect of protein levels on growth performance of abalone (Mai *et al.*, 1995).

Dietary protein (%)	Weight gain (mg/abalone)	
	<i>H. tuberculata</i>	<i>H. discus hannai</i>
0.28	44.6±0.4 <sup>a</sup>	-14.7±0.6 <sup>a</sup>
10	202±13.7 <sup>a</sup>	284±7.0 <sup>a</sup>
20	312±9.6 <sup>b</sup>	486±44.3 <sup>bc</sup>
25	313±29.6 <sup>cb</sup>	567±39.2 <sup>c</sup>
30	359±29.7 <sup>c</sup>	523±7.0 <sup>bc</sup>
35	249±24.8 <sup>c</sup>	507±19.9 <sup>bc</sup>
40	364±4.90 <sup>c</sup>	444±32.7 <sup>b</sup>
50	244±7.40 <sup>ab</sup>	489±14.8 <sup>bc</sup>

Mean values in the same column, sharing the same superscript was not significant difference ( $P > 0.05$ ).



**Figure 4.1.** Relationship between weight gain and dietary protein level for abalone *H. discus hannai* (above curve) and *H. tuberculata* (second lower curve) as described by polynomial regression (Mai *et al.*, 1995).

Similarly Coote *et al.* (2000) also used casein and gelatin supplemented with crystalline amino acids (Arg, Thr and Met) to simulate the amino acid profile of soft body abalone. To obtain the optimal dietary protein level, the juvenile abalone *H. laevigata* were fed dietary protein ranging from 12 % to 46 % (Table 4.2). Statistical analysis demonstrated that there was no significant difference in specific weight gain between 24 % to 46 % protein levels in test diets. Second-order polynomial regression analysis of specific growth rate also confirmed the maximal growth of abalone when animals were fed dietary protein level of 27 %. These results were very similar to those of Mai *et al.* (1995) and suggest a broad protein requirement.

**Table 4.2.** Weight gain of abalone fed various dietary protein levels (Coote *et al.*, 2000).

Dietary protein level (%)	SGR (%day <sup>-1</sup> )	Weight gain (g)
12.2	10.5 <sup>ac</sup>	0.61 <sup>ce</sup>
18.8	12.6 <sup>a</sup>	0.85 <sup>ae</sup>
23.9	11.3 <sup>ac</sup>	0.88 <sup>ae</sup>
27.4	9.6 <sup>ac</sup>	1.20 <sup>a</sup>
29.4	11.4 <sup>ac</sup>	1.10 <sup>ab</sup>
32.2	9.60 <sup>ac</sup>	0.91 <sup>ae</sup>
35.3	9.3 <sup>ac</sup>	1.07 <sup>ad</sup>
37.4	10.0 <sup>ac</sup>	0.74 <sup>bde</sup>
39.5	8.3 <sup>bc</sup>	0.81 <sup>ae</sup>
41.8	8.3 <sup>bc</sup>	0.77 <sup>bde</sup>
46.1	7.7 <sup>bc</sup>	0.73 <sup>bde</sup>

Note: Mean values in the same column, sharing the same superscript was not significant difference ( $P > 0.05$ ).

In another study by Sales *et al.* (2003), they also attempted to use diets with an amino acid profile similar to the abalone soft-body tissue by using casein combination with fishmeal without supplementation of crystalline amino acids. The juvenile abalone *H. midae* (about 5g) were offered various dietary protein levels ranging from 5.5 % to 48 %. Statistical analysis revealed that there was no significant difference in weight gain of abalone when they fed with diets containing protein ranging from 28.6 % to 47.9 % (Table 4.3). However, the second-order polynomial regression analysis showed that the protein at which produced a maximum body weight gain was at 35.9 %.

**Table 4.3.** Weight gain of abalone fed various dietary protein levels (Sales *et al.*, 2003).

Dietary protein level (%)	Weight gain (g)
5.5	1.12 <sup>a</sup>
13.4	2.58 <sup>b</sup>
21.8	3.59 <sup>c</sup>
28.6	4.14 <sup>d</sup>
39.4	4.13 <sup>d</sup>
47.9	3.82 <sup>cd</sup>

Note: Mean values in the same column, sharing the same superscript was not significant difference ( $P > 0.05$ ).

In contrast, several studies did not attempt to mimic the amino acid of their diets with amino acids profile of animal's tissue (Britz 1996; Gomez-Montes *et al.*, 2003; Bautista-Teruel *et al.*, 2003). Britz (1996) used semi-purified diets without supplementation of crystalline amino acids and did not consider the hypothesis of matching amino acids of diets and soft body tissue. The juvenile abalone *H. midae* (about 1.5g) were offered various dietary protein levels ranging from 27 % to 47 %. The results showed that the growth rate of abalone was correlated to the increase protein level, and statistically there was no significant difference in weight of abalone that were fed dietary protein ranging from 32 % to 47 % protein (Table 4.4).

**Table 4.4.** Growth performance of abalone fed different dietary protein (Britz *et al.*, 1996).

Dietary protein level (%)	Final weight (g)
27	2.04 <sup>b</sup>
32	2.14 <sup>ab</sup>
37	2.34 <sup>ab</sup>
42	2.44 <sup>ab</sup>
47	2.64 <sup>a</sup>

Initial weight was 1.46g; Mean values in the same column sharing the same superscript was not significant difference ( $P > 0.05$ ).

Similar to Britz, Gomez-Montes *et al.* (2003), used a practical diet without supplementation of crystalline amino acids mixing fishmeal and soybean protein isolated. The juvenile abalone *H. fulgens* (0.2 g) were fed five different dietary protein levels of 25.8 %, 30.9 %, 34.9 %, 40.5 %, and 44.1 %. The results showed that specific growth rate of abalone was significant difference higher at dietary protein levels up to 44 % (Table 4.5). It was not clear at which protein level that produced an optimal growth rates for abalone in this study because of a linear relationship between dietary protein and SGR.

**Table 4.5.** Growth response of abalone to various protein:energy (Gomez-Montes *et al.*, 2003).

Protein:energy	SGR (%/day)
62 (25.8% protein)	1.28 <sup>d</sup>
74 (30.9)	1.50 <sup>b</sup>
85 (34.9)	1.86 <sup>b</sup>
100 (40.5)	2.43 <sup>a</sup>
108 (44.1)	2.52 <sup>a</sup>

Values in the same column, sharing the same superscript was not significant difference ( $P > 0.05$ ) among diets.

The protein requirements studies for abalone were summarized in the Table 4.6. The protein requirements of abalone in both Britz (1996) and Gomez-Montes *et al.* (2003) were higher than those reported in the previous studies (Mai *et al.*, 1995; Coote *et al.*, 2000; Sales *et al.*, 2003). This was due to a mismatch between feed and tissue amino acids and the abalone may simply metabolize excess amino acids. On the other hand, mismatch in essential amino acids profile of feed and essential amino acids (EAA) of animal tissue could lead to poor growth in abalone. For example, poor growth was obtained when abalone were fed with formulated diet containing amino acid profile that do not match animals tissue and contained only plant protein sources of soybean and *Spirulina* (Bautista-Teruel *et al.*, 2003). Moreover, it is clearly indicated that significant lower growth rates when abalone were fed with dried kelp *Ecklonia maxima* (Naidoo *et al.*, 2006) or *Laminaria* (Cho *et al.*, 2008). Therefore, these studies prove the hypothesis that the growth rate supported and related to the degree of the amino acid profile of

feed matches the amino acid profile of tissue. While the knowledge of matching on EAA profile of abalone diet and animal tissue is fairly well known, there is still limited information on feed intake consideration that was not measure by these studies.

**Table 4.6.** A summary of the protein requirement study for abalone.

No.	Abalone species	No statistically difference between protein levels (%)	Optimal protein level (%)	Reference
1	<i>H. tuberculata</i>	25-40	32	Mai <i>et al.</i> , 1995
	<i>H. discus hannai</i>	20-50	35	
2	<i>H. laevigata</i>	24-46	27	Coote <i>et al.</i> , 2000
3	<i>H. midae</i>	29-48	36	Sales <i>et al.</i> , 2003
4	<i>H. midae</i>	32-47	47	Britz 1996
5	<i>H. fulgens</i>	40-44	44	Gomez-Montes <i>et al.</i> , 1996

In study to be described, beside focusing on the matching amino acid profile of diet and amino acids profile of opihi soft body tissue, feed consumption rate was also documented. We speculated feed intake that would also be responsible for the difference in growth performances of opihi. The growth performance of opihi was observed when they were fed with different protein levels from 21 % to 50 % in two experimental trials. These diets were formulated by employing the best performance diet of fish/soy/krill, without supplementation of crystalline amino acids.

## MATERIALS AND METHODS

Experimental diets. Table 4.7 shows the experimental diets used for this study. Two protein trials were conducted. For the protein trial 1, five different dietary protein levels of 27 %, 32 %, 37 % 42 % and 47 % were tested for 90 days. Carbohydrate and lipid levels were kept constantly at approximately 18.0 % and 4.97 %, respectively. A package of protein ingredients of fish meal, defatted soybean meal and krill meal was used as a base because these ingredients were found to be the most well performance in both palatability and supported well sustain life

and good growth (Hua and Ako 2012). Besides *Porphyra*, krill meal was the most attractive one. Wheat flour was used as starch and diatomaceous earth was used to balance in the diets as protein source was decreased. Protocols for diet preparation were described by previous chapter. Wheat flour and alginate were gelatinized in boiling water before being mixed with other ingredients.

Based on the results of the trial 1, a following protein feeding trial was repeated with four different dietary protein levels consisting of 21 %, 30 %, 35 % and 50 % with lower constant carbohydrate level of about 11.0%. Lipid level was maintained at 5.13 %. The animals were fed for over 60 days.

**Table 4.7.** Composition of formulated diet (% dry matter).

Ingredient	Dietary protein -Trial 1					Dietary protein-Trial 2			
	27%	32%	37%	42%	47%	21%	30%	35%	50%
Fishmeal	16.5	19.5	22.5	25.5	28.5	13.4	17.0	21.0	30.4
Defatted soybean	11.5	14.5	17.5	20.5	23.5	11.0	12.7	16.6	24.4
Krill meal	4.50	7.50	10.5	13.5	16.5	7.10	8.00	11.0	16.1
Porphyra *	14.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0
Wheat flour	15.4	14.3	13.3	12.2	11.1	8.98	5.30	4.30	0.80
Diatomaceous earth	29.2	21.9	14.6	7.30	0.00	36.8	35.2	25.8	7.30
Alginate	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Corn/fish oil**	2.50	1.90	1.20	0.60	0.00	2.32	1.40	0.90	0.60
Vit. mix***	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Total	100	100	100	100	100	100	100	100	100
Water	100	100	100	100	100	133	133	133	133
Calculated nutrient									
Protein	26.5	31.7	37.0	42.4	47.7	21.2	30.5	35.8	49.2
Lipid	4.97	4.97	4.97	4.97	4.97	5.13	5.13	5.13	5.13
Carbohydrate	17.5	17.8	18.1	18.3	18.6	11.0	11.0	11.2	11.4

\*This is commercial seasoned seaweed known as nori or the red algae

*Porphyra tenera* or *yezoensis*. Nishimoto Trading Co. Ltd., Korea

\*\* Mixture of corn oil and menhaden oil (1:1; v/v)

\*\*\* Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute).

Because of our hypothesis that previous studies had showed wide spreads in optimal protein levels due to inappropriate amino acid balance for abalone, we determined the amino acid profiles of opihī tissue in order to have an optimal balance in our trials. The amino acid profiles of ophi tissue and of the dietary protein in trial 1 were determined by Dr. Ju at the Aquatic Feed and Nutrition Laboratory, Oceanic Institute, Hawaii, USA, according to the

described method of Ju *et al.* (2008), while the amino acid profile of the experimental dietary protein trial 2 were calculated “as fed”. The results are presented as A/E ratio (Table 4.8). Most of the essential amino acids of diets were identical and/or close to the amino acid profile of opihi tissue except for Arg and Thr which were lower in the experimental diets compared to tissue.

**Table 4.8.** The A/E ratio [(each EAA/Total EAA) x 1000] amino acids of the dietary protein and opihi tissue.

Essential AA	Opihi tissue	*Protein trial 1					**Protein trial 2			
		270	320	370	420	470	210	300	350	500
Arg	224	123	118	129	125	139	208	198	190	173
His	33.8	39.5	37.4	41.5	39.9	44.6	29.1	29.4	29.8	32.3
Ile	81.4	88.7	89.5	87.7	88.6	99.5	80.4	80.9	81.2	80.8
Leu	146	158	157	155	156	177	149	148	147	142
Lys	69.2	159	170	149	160	178	110	112	114	116
Met/Cys	68.3	74.1	75.1	77.6	77.7	87.0	51.2	53.5	55.4	57.4
Phe/Tyr	123	158	161	159	160	179	187	194	200	219
Thr	136	97.9	92.0	97.8	93.4	98.0	78.9	79.1	79.3	78.4
Val	117	103	99.9	103	99.8	111	107	105	104	100

\* analyzed by Dr. Ju (Oceanic Institute)

\*\* values are calculated “as fed”

Experimental animals. Animals ( $3.12 \pm 0.86$  g) used for this study were collected from remote shoreline areas in Oahu Island, Hawaii, USA. After collection the animals were immediately transferred to the laboratory and placed in aquaria with plastic liners, with algae seeded with benthic diatoms from Hawaii Institute of Marine Biology. An aquarium biofilter system ( $586 \text{ L h}^{-1}$ ) was used to simulate water movement. They were held for about 3 days of acclimation. Thereafter, nine opihi were selected for each experimental group and their initial weight and shell length were measured. For the first trial, each individual was placed into its own plastic colander (20 cm diameter). The colanders were put in tubs, 9 colanders per tub. Five tubs were used for the first protein trial. Animals that died in the first three days after placement

into the test system were replaced. We observed a few more mortalities due to removing the animals from the colanders for weighing. Therefore, softer containers made of soft screen polyethylene plastic, cone shaped, 13 cm diameter, 17 cm height, were used in the second protein trial. The containers were placed on a tray in tubs (150 L), nine containers per tub. Four tubs were used, connected to a reservoir aquarium with particle filters, and a submerged water pump was used to recycle water at a flow rate of  $3 \text{ L min}^{-1}$  from the reservoir aquarium. Water temperature ranged from 24-26°C and salinity was maintained at 35 ppt.

It is noticed that, the collected opihi for the second protein trial were not held in the biofilm aquarium. They were held in a holding aquarium without algae seedless from HIMB and being used for the experiment in the day after collection. There was about 18% mortalities for three patches of collection occurred in the first day after collection in the aquarium and about 41% occurred in the first three days after putting them into the test system. These were replaced with new animals and eventually the animals were stable in the test system. We did an over optimistic in the mortality rate in previous study, which was about only 30 % in three days after collection when the animals were held in biofilm aquaria, presumably due to injury from collection.

Feeding regime and feeding evaluation were as in our previous description chapter. The animals were fed one time per day, at 18:00 daily, as animals did not eat during the day. A square piece of feed ( $1.0 \text{ cm}^2/\text{piece}$ ; approximately 130 mg mean weight; 48.5 % dry weight) was given in the evening to each individual animal by placing the feed on the side of the container. Feeding amount was judged in each individual container the next morning. The percentage of the squares fed that was eaten, was estimated and converted to dry weight for each individual. In some cases, the left over feed was siphoned and dried in the oven for 5 h and re-weighed to obtain the weight of uneaten feed. Feed consumption data were also converted to percentage dry matter per bodyweight per day (%DW/BW/day). It was noticed that the animals normally did not eat well for the first few days after putting them into the test system, therefore feeding rate of animals for three days after stocking and three days after replacement were not counted in our calculations. With this taken into consideration, as animals were conditioned to the artificial feed used in the feed trials, the following data was calculated:

Feed consumption (% dry matter/ soft body weight/day) for individual = [feed eaten (g, dry weight)/ soft body weight (g)] x 100

Protein intake = total feed intake (g/individual) x (g protein/g dry feed)

Sampling and analytical methods. The growth of animals in weight (g) and shell length (cm) were measured monthly. The growth was expressed in terms of specific growth rate (SGR), weight gain and shell length increase. The shell length was measured with a dial caliper caliper (0.01 inch) and weight determined with an electronic scale (0.01 g error) every 4 weeks

Mean weigh gain (g/individual) =  $W_f - W_i$

Weight gain (WG, %) =  $[(W_f (g) - W_i (g)) / W_i (g)] \times 100$

Specific growth rate (SGR) =  $\{(\ln W_f - \ln W_i) / T\} \times 100$ ,

where  $W_f$  is final weight,  $W_i$  is initial weight and T is total day of the experiment.

Feed conversion ratio (FCR) = total feed eaten (g)/wet weight gain (g).

Protein efficiency ratio = Wet weight gain (g)/protein intake (g)

Solid leaching of diets. Weight matter leaching was examined for dietary protein trial 2.

The experiment was conducted in an experimental test system. Three pieces of each feed (1.0 cm<sup>2</sup>/piece) served as for replication, three replications per diet. They were weighed to obtain the initial weights of the three replications, then placed in an empty colander without opihi for 12 h. Stability of feed was assessed according to the equation: Water absorbed (%) =  $\{(FW - IW) / IW\} \times 100$ . Where IW is initial weight of feed, and FW is the final weight of feed of the same diet after being immersed in water. The retained feed was rinsed with fresh water and transferred to a drying oven for 12 hr at 100°C to obtain dry weight. To obtain the initial dry weight of each diet, three other pieces of each diet were weighed and dried in the oven, this dry weight served as initial dried weight control. This data was used to determine leaching losses due to soaking.

Data analysis. Data from each treatment were subjected to One-way ANOVA, followed by Tukey test was used to test the normalized weight gain (g), specific growth rate (% body weight/day), feed consumption rate (% dry matter/body weight/day) and feed conversion ratio (dry feed eaten/weight gain) using SPSS (SPSS Inc., Chicago, IL). The optimal protein requirement of opihi would also be estimated by second-order polynomial regression analysis model (Lowell 1989; Mai *et al.*, 1995).

## RESULTS

Dietary protein trial 1. Table 4.9 shows the growth performance of opihi fed at 27% dietary protein level over 90 days. Among nine tested opihi, two animals (animal 2, 3) died

shortly after weighing and one (animal 5) died during the experiment for an unknown reason. Numbers for these animals were not counted. The final survival was 67 %. The weight gain of animal 9 was lower than one standard deviation from the mean and was eliminated from consideration. Thus, the average normalized weight gain was  $0.31\pm 0.11$ g. The specific growth rate was  $0.30\pm 0.12\%$  BW/day. The average feed consumption rate and FCR were  $0.9\pm 0.14\%$  DM/BW/day and  $0.94\pm 0.8$ , respectively. It is noted that the FCR was calculated without normalization of weight gain.

**Table 4.9.** Growth trial parameters of animals fed dietary 27 % protein.

	Animal No.									Mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	3.16	4.10	2.93	3.09	3.44	3.36	4.89	2.57	3.05	3.40±0.70
Day 30	3.18	4.10	2.94	3.11	3.51	3.46	5.02	3.07	3.05	3.55±0.70
Day 60	3.53			4.12		4.11	6.01	3.54	2.82	4.26±1.02
Day 90	3.67			4.38		4.42	6.05	3.64	3.11	4.43±0.93
Weight gain (g)	0.51			1.24		1.06	1.16	1.07	0.06	1.02±0.30
SGR	0.17			0.39		0.30	0.44	0.19	0.02	0.30±0.12
Feed consump.	0.72			0.97		0.91	0.81	1.07	0.80	0.90±0.14
FCR	1.41			0.75		0.86	0.70	1.00		0.94±0.80

Growth performance of animals that were fed with dietary of 32 % protein was shown in the Table 4.10. Three animals (animal 3, 4 and 7) died during the experiment, one of them was dead right after the first measurement because of weighing. The final survival was 67 %. No dead animals were included in calculations. Similar to diet 27 %, the growth record of animal 9 was unusual with the weight was just constant for the first month and reduced in the second month. Only animal 1, 2, 5, 6 and 8 were used for calculation because animal 9 was considered an outlier. Thus, the average normalized weight gain was  $0.37\pm 0.13$ g. The average of SGR ( $0.35\pm 0.11\%$  BW/day), feed consumption rate ( $0.9\pm 0.16\%$  DM/BW/day), and the average FCR ( $0.94\pm 0.24$ ) were reported.

**Table 4.10.** Growth trial parameters of animals fed dietary 32 % protein.

	Animal No.									Mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	3.32	3.25	3.12	3.11	2.52	3.01	3.01	2.05	2.18	2.84±0.47
Day 30	3.62	3.05	3.32	3.18	2.52	3.05	3.05	2.25	2.18	3.01±0.43
Day 60	4.60	3.80			3.36	3.54		2.18	2.25	3.50±0.88
Day 90	4.74	3.86			3.67	3.89		3.06	2.30	3.84±0.60
Weight gain (g)	1.42	0.61			1.15	0.88		1.01	0.12	1.01±0.30
SGR	0.40	0.19			0.42	0.28		0.45	0.06	0.35±0.11
Feed consump.	0.80	0.68			0.96	0.98		1.09	1.17	0.91±0.16
FCR	0.56	1.11			0.83	1.11		1.01		0.94±0.24

Table 4.11 shows the growth performance of opihi fed at 37 % dietary protein level over 90 days. Among eight tested opihi, four animals (animal 2, 3, 6 and 7) died during the experiment; two of the dead animals were due to weighing and were not counted for calculation. The other non-survivors were not counted as well. The final survival was 50 %. The average normalized weight gain was  $0.41 \pm 0.20$ g. The animal 1 did not count because of the average weight gain of animal 1 was lower than one standard deviation from the mean. The SGR was  $0.41 \pm 0.1$ % BW/day. The average feed consumption rate was  $1.05 \pm 0.13$ % DM/BW/day and average FCR was  $1.23 \pm 0.53$ .

**Table 4.11.** Growth trial parameters of animals fed dietary 37 % protein.

	Animal No.								Mean±SD
	1	2	3	4	5	6	7	8	
Initial weight (g)	3.87	3.15	1.01	2.06	3.19	2.02	3.62	1.50	2.55±1.05
Day 30	3.92	3.18	1.11	2.16	3.29	3.11	3.72	1.90	2.84±0.77
Day 60	3.81			2.11	4.18			2.37	2.89±1.13
Day 90	4.12			2.5	4.5			2.41	3.38±1.18
Weight gain (g)	0.25			0.70	1.31			0.91	0.91±0.31
SGR (%BW/day)	0.07			0.25	0.38			0.53	0.41±0.10
Feed consump.	0.58			1.12	0.91			1.13	1.05±0.13
FCR				1.75	0.69			1.24	1.01±0.53

Table 4.12 showed the growth performance of opihi fed at 42 % dietary protein level over 90 days. Among eight tested opihi, three animals (animal 2, 3 and 7) died during the experiment and were not further considered. The final survival was 63 % in the course of experiment. The average normalized weight gain was 0.24±0.15g. Animal 1 did not count because of the growth record of animal 1 was unusual with the average weight gain lower than one standard deviation from the mean. The average specific growth rate was 0.32±0.10%BW/day and feed consumption was monitored of 0.81%DM/BW/day. The FCR was 1.11.

**Table 4.12.** Weight measurement of animal fed dietary of 42 % protein.

	Animal No.								Mean±SD
	1	2	3	4	5	6	7	8	
Initial weight	2.41	3.15	2.45	2.92	2.49	1.65	3.15	2.68	2.61±0.49
Day 30	2.48	3.15	2.65	2.49	2.49	1.85		2.78	2.40±0.39
Day 60	2.30			3.16	2.52	1.65		2.93	2.57±0.66
Day 90	2.55			4.29	3.01	2.31		3.43	3.26±0.82
Weight gain (g)	0.14			1.37	0.52	0.66		0.75	0.83±0.38
SGR (%BW/day)	0.06			0.43	0.21	0.37		0.27	0.32±0.10
Feed consump. (%DM/BW/day)	0.82			0.80	0.74	0.93		0.78	0.81±0.08
FCR				0.58	1.42	1.40		1.04	1.11±0.34

Growth performance of animal fed with dietary of 47 % protein is shown in the Table 4.13. Four animals (animal 2, 4, 5 and 7) died during the experiment and were not counted for calculation. Some mortalities (2 animals) occurred right after weighing. The final survival was 56 %. The growth record of animal 1 was unusual. Only animal 3, 6, 8 and 9 were used for calculations because the average weight gain of animal 1 was lower than one standard deviation from the mean. The average normalized weight gain and SGR were  $0.24\pm 0.08\text{g}$  and  $0.23\pm 0.07\%\text{BW/day}$ , respectively. The feed consumption was low at level of  $0.56\pm 0.12\%\text{DM/BW/day}$  and the FCR was high at level of  $1.12\pm 0.25$ .

**Table 4.13.** Growth trial parameters of animals fed dietary 47 % protein.

	Animal No.									Mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight	3.27	3.10	2.56	2.15	3.15	2.18	2.64	2.16	2.01	2.58±0.49
Day 30	3.47	3.36	2.68	2.20	3.55	2.68	2.74	2.56	2.51	2.70±0.41
Day 60	3.37		3.38			2.42		2.57	2.51	2.72±0.44
Day 90	3.31		3.46			2.55		2.57	2.48	2.87±0.47
Weight gain (g)	0.04		0.90			0.37		0.41	0.47	0.54±0.25
SGR	0.01		0.33			0.17		0.19	0.23	0.23±0.07
Feed consump.	0.54		0.73			0.52		0.48	0.50	0.56±0.12
FCR			0.82			1.41		1.18	1.07	1.12±0.25

Dietary protein trial 2. Table 4.14 shows the growth performance of opihi fed with 21 % dietary protein level over 60 days. Among nine tested opihi, one animal (animal 5) died after Day 25. This animal was not counted. The final survival was 89 %. The weight gain of animals 1 and 9 were lower than one standard deviation from the mean and was eliminated from consideration. Thus, the average weight gain was 0.56±0.20 g. The mean specific growth rate was 0.19±0.09 %BW/day.

**Table 4.14.** Growth trial parameters of animals fed dietary 21% protein.

	Animal No.									Mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	7.70	7.32	3.97	4.13	4.26	4.16	4.85	3.01	5.63	5.00±1.59
Day 25	7.55	7.39	3.86	4.35	4.32	3.94	4.98	2.85	5.64	4.99±1.60
Day 60	7.73	8.31	4.42	4.85		4.43	5.41	3.39	5.58	5.52±1.69
Weight gain (g)		0.99	0.45	0.72		0.27	0.56	0.38		0.56±0.20
Feed consump.		0.36	0.49	0.34		0.41	0.59	0.6		0.47±0.10
FCR		0.36	1.09	0.47		1.52	1.05	1.58		1.01±0.51

Growth performance of animals that were fed with dietary of 30 % protein was shown in the Table 4.15. The final survival was 100 %. The weight gain of animals 1 and 4 were lower than one standard deviation from the mean and was eliminated from consideration. Thus, the average weight gain was  $0.77\pm 0.40$  g. The mean specific growth rate was  $0.22\pm 0.13$  %BW/day.

**Table 4.15.** Growth trial parameters of animals fed dietary 30 % protein.

	Animal No.									Mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	11.1	6.50	5.31	3.95	6.10	4.61	4.80	5.30	5.69	5.93±2.09
Day 25	10.9	6.57	5.11	3.64	6.14	4.83	4.62	5.31	5.91	5.89±2.06
Day 60	11.1	7.17	6.4	4.16	6.48	5.73	5.31	5.61	7.02	6.55±1.94
Weight gain (g)	0.00	0.67	1.09	0.21	0.38	1.12	0.51	0.31	1.33	0.77±0.40
Feed consump.		0.37	0.41	0.45	0.32	0.46	0.48	0.49	0.20	0.41±0.16
FCR		0.55	0.38	2.14	0.84	0.41	0.94	1.58	0.15	0.87±0.68

Growth performance of animals that were fed with dietary of 35 % protein was shown in the Table 4.16. The final survival was 89 %. The weight gain of animals 1 and 7 were lower than one standard deviation from the mean and were eliminated from calculation. The average weight gain was  $1.00\pm 0.44$  g. The mean specific growth rate was  $0.28\pm 0.12$  %BW/day.

**Table 4.16.** Growth trial parameters of animals fed dietary 35 % protein.

	Animal No.									Mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	3.56	3.83	12.1	5.95	4.44	4.70	5.01	4.58	5.89	5.56±2.58
Day 25	3.47	3.85	12.0	6.09	4.93	4.25	4.38	5.20	6.04	5.57±2.54
Day 60	3.74	4.32	13.0	7.00	5.09		4.72	6.33	7.02	6.41±2.94
weight gain (g)		0.49	0.90	1.05	0.65			1.75	1.13	1.00±0.44
Feed consump.		0.29	0.15	0.35	0.35			0.55	0.36	0.40±0.17
FCR		0.59	0.16	0.33	0.54			0.31	0.32	0.38±0.16

Growth performance of animals that were fed with dietary of 50 % protein was shown in the Table 4.17. The final survival was 89 %. The weight gain of animals 1 was lower than one standard deviation from the mean and was eliminated from consideration. Thus, the average weight gain was  $0.38 \pm 0.10$  g. The mean specific growth rate was  $0.13 \pm 0.04$  %BW/day.

**Table 4.17.** Growth trial parameters of animals fed dietary 50 % protein.

	Animal No.									Mean $\pm$ SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	5.12	4.13	4.01	6.62	4.45	4.81	5.21	5.12	6.70	5.17 $\pm$ 0.98
Day 25	4.82	3.96	3.66	6.69	4.65	4.96	5.62		6.71	5.13 $\pm$ 1.14
Day 60	4.98	4.4	4.38	6.89	4.88	5.31	5.72		7.04	5.45 $\pm$ 1.03
Weight gain (g)		0.27	0.37	0.27	0.43	0.50	0.51		0.34	0.38 $\pm$ 0.10
Feed consump.		0.32	0.31	0.20	0.26	0.30	0.28		0.31	0.27 $\pm$ 0.06
FCR		1.19	0.84	0.74	0.60	0.60	0.55		0.91	0.78 $\pm$ 0.22

The data of growth performance of opihi fed with different dietary protein levels is summarized in the Table 4.18. The mean of initial weight, weight gain, SGR, feed consumption, FCR and survival rates are obtained from the Table 4.9 to 4.17. For the first protein trial, statistical analysis by One-way ANOVA followed by Tukey Kramer showed that there was no significant ( $P > 0.05$ ) effect of various protein levels (27-47 %) on the weight gain and the specific growth rates of animals, although this dropped off insignificantly at higher protein levels from 42 % to 47 % diets over 90 days.

A low survival rate in the first protein trial could affect the strength of results. Mortality (25-50 %) was due in part to handling during weighing when the animals were removed from the colander, and was not affected by dietary protein. They died after weighing, we assume due to physical injury and/or stress. A higher survival rate was obtained in protein trial 2 using the softer containers, ranging from 89 % to 100 %.

There was a significant ( $P < 0.05$ ) difference in weight gain and specific growth rates of animals in the second dietary protein trial over 60 days, possibly due to better methods and more practice. Weight gain tracked specific growth rates was progressively increased to maximum at

35 % protein diet, and significantly higher than those animals fed with 50 %, but did not differ significantly compared to the 21 % and 30 % diets.

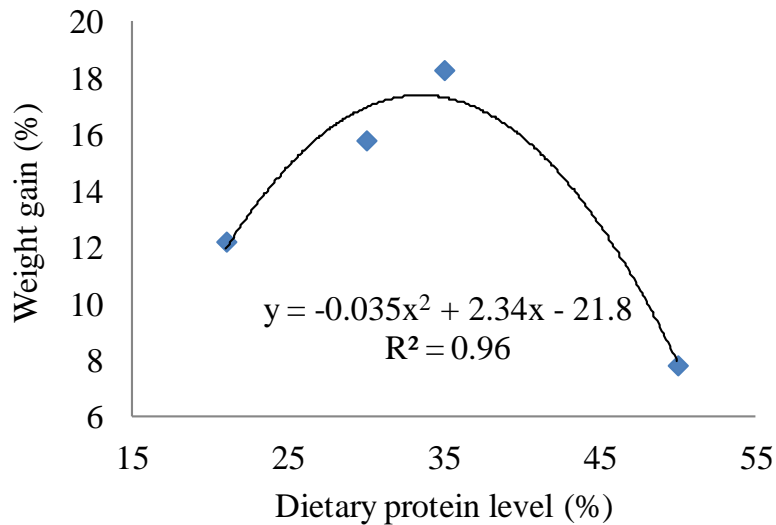
There was a significant difference in feed consumption of animals. Feed consumption for animals that were fed with 50 % dietary protein was significantly lower ( $P < 0.05$ ) than those animals that were fed with dietary protein of 21 %, 30 % and 35 % in the second protein trial. The highest feed efficiency ratio tracked the weight gain of those animals that were fed with the 35 % dietary protein and was significantly higher than 50 % diet.

**Table 4.18.** Effect of dietary protein on growth performance, specific growth rate (SGR), feed consumption, protein efficiency ratio (PER) and survival of opihi *C. sandwicensis*.

	Dietary protein (g kg <sup>-1</sup> )	Initial weight (g opihi <sup>-1</sup> )	Weight gain (g opihi <sup>-1</sup> )	SGR (%day <sup>-1</sup> )	Feed consumption (%DM/BW/day)	PER	Survival (%)
Trial 1	270	3.35±0.79 <sup>a</sup>	1.02±0.30 <sup>a</sup>	0.30±0.12 <sup>a</sup>	0.90±0.14 <sup>a</sup>	4.29±1.03 <sup>a</sup>	67
	320	2.72±0.54 <sup>a</sup>	1.01±0.30 <sup>a</sup>	0.35±0.11 <sup>a</sup>	0.91±0.16 <sup>a</sup>	4.54±1.45 <sup>a</sup>	75
	370	2.65±1.01 <sup>a</sup>	0.97±0.31 <sup>a</sup>	0.41±0.10 <sup>a</sup>	1.05±0.13 <sup>a</sup>	4.23±1.52 <sup>a</sup>	50
	420	2.43±0.48 <sup>a</sup>	0.83±0.38 <sup>a</sup>	0.32±0.10 <sup>a</sup>	0.81±0.08 <sup>ab</sup>	4.26±1.03 <sup>a</sup>	63
	470	2.44±0.51 <sup>a</sup>	0.54±0.25 <sup>a</sup>	0.23±0.07 <sup>a</sup>	0.56±0.12 <sup>b</sup>	3.95±0.45 <sup>a</sup>	56
Trial 2	210	5.00±1.59 <sup>a</sup>	0.56±0.26 <sup>ab</sup>	0.19±0.09 <sup>ab</sup>	0.46±0.20 <sup>a</sup>	5.70±2.72 <sup>ab</sup>	89
	300	5.93±2.09 <sup>a</sup>	0.77±0.40 <sup>ab</sup>	0.22±0.13 <sup>ab</sup>	0.41±0.16 <sup>a</sup>	6.20±3.78 <sup>a</sup>	100
	350	5.56±2.58 <sup>a</sup>	1.00±0.44 <sup>a</sup>	0.28±0.12 <sup>a</sup>	0.40±0.17 <sup>a</sup>	6.79±1.41 <sup>a</sup>	89
	500	5.17±0.98 <sup>a</sup>	0.38±0.10 <sup>b</sup>	0.13±0.04 <sup>b</sup>	0.28±0.13 <sup>b</sup>	2.47±0.75 <sup>b</sup>	89

<sup>a,b</sup> Means, within the column, different superscript letters are significant differently ( $P < 0.05$ ) in the same trial.

The growth response of opihi in terms of weight gain (%) of animals in dietary protein trial 2 was fitted into quadratic models (Fig. 4.2). The best fit for the estimation of optimal protein level could be described as  $Y = -0.035x^2 + 2.34x - 21.8$  ( $R^2 = 0.96$ ). The trend of growth showed that maximum weight gain appeared to be about 35 % dietary protein.



**Figure 4.2.** Relationship between weight gain and dietary protein level of the second trial 2 for *C. sandwicensis* for 60 days.

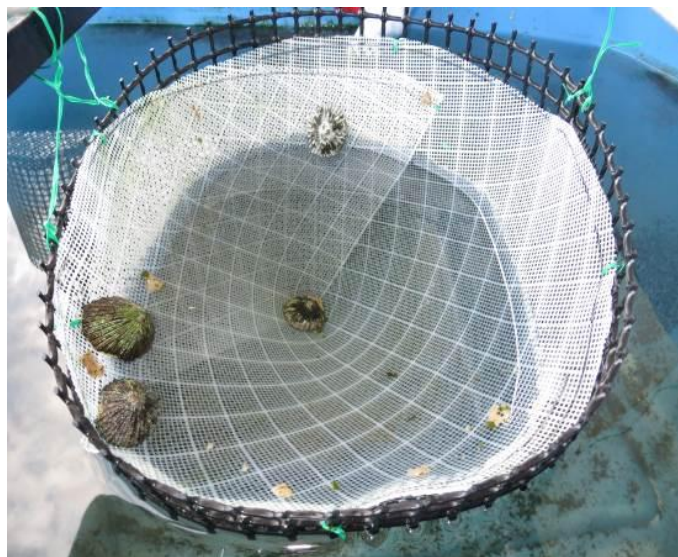
The percentage of solids remaining among the diets was  $98.0 \pm 1.13$  %,  $96.8 \pm 1.39$  %,  $97.8 \pm 1.17$  % and  $98.5 \pm 0.32$  % for 21, 30, 35 and 50 % of dietary protein, respectively. Statistical analysis for solid leaching rates showed that there was no significant difference ( $P > 0.05$ ) in percentage of remaining dry weight between the dietary protein levels in trial 2.

## DISCUSSION

The purpose of this chapter was to determine the optimum dietary protein level, which produces the maximum growth of opihi. There have been no studies on nutrient requirement in opihi, but the protein requirements and other nutrients factors have had been well studied for abalone. In our previous study showed that diet containing fishmeal, soymeal with krill meal and *Porphyra* was well consumed and produced a reliable growth rate in opihi. This diet was continuously used as a base diet in this study.

The high mortality rate in our first trial raised questions about the statistical strength of our findings about the optimum protein requirement for opihi in that trial. About half of mortalities in 27 % (2 animals out of 3 mortalities), 37 % (2 animals out of 4 mortalities) and 47 % (2 animals out of 4 mortalities) treatments was due to weighing because a mistake was made due to the thought that opihi may be easily remove them from the colander at night time while they was

feeding. We did not realize that how we could have avoided about half the mortalities while being experimental process and until finished the test. The mortality in this study suggests that colanders are not a good holding facility for handling opihi. Another kind of soft container was designed to overcome the colander problems. It was a cone made of soft polyethylene plastic (Fig. 4.3) and was used in the second protein feed trial. Animals were removed easily for weighing during the course of experiment by deforming the soft plastic screen. This method improved survival rates significantly to 89-100 %, improving the statistical strength of the results of our second trial.



**Figure 4.3.** Soft polyethylene plastic containers with cones-shaped for holding animal

The results of this study showed that the growth of opihi did not correlate to increased dietary protein levels. No significant effect of various dietary protein levels on weight gain and SGR of opihi, statistical analysis by One-way ANOVA showed that there was no significant difference ( $P > 0.05$ ) in weight gain of opihi among the dietary protein ranging from 27 % to 47 % protein. As stated above, no statistically significant difference in growth rates among the diets may be due to a low survival of animals. Therefore, it is premature to draw any conclusion from the Trial 1 and the data may be use as a notification for following trial. Better and significant data were obtained in Trial 2 (Table 4.18, Fig. 4.2). The highest weight gain was achieved with the 35 % protein diet, but there was no significant difference in weight gain with those animals

fed with 21 and 30 % diets. This indicated that opihi could satisfy their needs with a low protein level above 21 %. The trend of the growth rate suggests that when the protein level is too high it may be detrimental to growth. In fact, the results from the 50 % protein group in the second trial showed poor growth, significantly lower than the other diets, indicating that excess protein is not easily metabolized by opihi.

The second-order polynomial regression model is a way graphical model which allows us to choose an optimal protein level among statistically insignificant differences. In our present study, the growth rate of opihi with increasing dietary protein ranged from 21 % and reached to the maximal level at approximately 35 % protein. The 35 % protein is the choice by polynomial analysis although it is not statically different from 21 % and 30 % diets (Fig. 4.2). The pattern of protein requirement for opihi in our study was similar to other studies for abalone species. The use of second-order polynomial analysis to estimate the optimal protein level for maximum growth of abalone *H. discus hannai* was about 35.6 % dietary protein while statistical analysis revealed there was no significant difference in weight gain of abalone that were fed dietary protein from 20.2 to 35.3 % and about 32.3 % for *H. tuberculata* within 20.2 to 40 % (Mail *et al.*, 1995), about 27 % (24-46 %) for *H. laevigata* (Coote *et al.*, 2000) and by Sales *et al.* (2003) for *H. midae* (36 %).

Feed consumption seemed to be responsible for growth rate. We have earlier found that feed consumption was correlated with the growth response of opihi (Hua and Ako 2012). Despite the matching amino acids of diets and animal tissue was concerned, but we thought that it is not sufficient to conclude the fact that supporting optimum growth for animal. Because the growth of animal involves in many factors such as feed intake. The feed intake tended to be positively related to growth rate of opihi. The lower feed consumption of animals that were fed 50 % corresponded to the decrease in the weight gain of animals. Overall, the feed consumption rates were low as compared to our previous study. On the other hand, a lower feed consumption rate in the second trial may be due to a lower carbohydrate level.

The specific growth rate of opihi ranged from 0.13-0.28 % BW/day for dietary protein trial 2. These growth rates are close to the growth rate (approximately 0.2-0.4% BW/day) for abalone *H. discus hannai* found by Uki *et al.* (1985), but lower compared to the growth rate (0.94-1.11% BW/day) of *H. midae* reported by Britz (1996). However, may not be valid to compare these values directly between opihi and abalone or even among the abalone species because the

apparent requirement may vary due to many different conditions such as difference in diets, experimental species, digestible protein, feed intake and the management of feeding regimes.

## CHAPTER 5 . THE EFFECT OF DIETARY CARBOHYDRATE LEVEL ON GROWTH PERFORMANCE OF OPIHI

### INTRODUCTION

The purpose of this study was to determine the optimal carbohydrate level for the growth of opihi. Dietary carbohydrate could act as good energy sources for abalone *H. kamtschatkana* (Taylor 1997), sparing the amount of protein needed. Providing an adequate amount of lipid or carbohydrate in the diet would minimize the use of expensive protein (National Research Council (NRC) 1993). Many studies have used lipid as an energy source and the optimal dietary protein to lipid ratio levels have been studied in fish (Satpathy *et al.* 2003; Alam *et al.* 2003), and abalone (Britz and Hetch 1997). For limpets, studies have showed that amylase activity was very high in the digestive system of the patellacean limpets including *Patella vulgate*, *Cellana radiata* and *Collisella pelta*, (Graham 1932, Balaparameswari 1975 and Jobe 1968, cited by Branch 1981), leading to the assumption that *C. sandwicensis* should also be able to utilize high levels of carbohydrate. On the other hand, lipase activity has not been found in limpets or opihi, and studies have shown that lipases were very low in digestive gland of abalone (Knauer *et al.*, 1996; Fleming *et al.*, 1996) suggesting a low capability of lipid utilization for opihi. We considered carbohydrate as a second macronutrient which needed to be examined. Carbohydrates are inexpensive and minimize the cost of opihi feed. Therefore, this study was conducted to examine the effect of various carbohydrate levels on growth performances of opihi.

There have been no studies on the dietary requirements for carbohydrate in opihi. Therefore we considered studies of carbohydrates requirements of abalone as a starting reference for opihi. The first report on the effect of various combinations of carbohydrate sources on growth performances of abalone used sodium alginate and dextrin in the test diet (Uki and Watanabe 1989). Abalone were fed with four diets; the first diet contained 20%:33% (sodium alginate:dextrin, v/v), the second was 30%:23%, the third diet of 40%:13% and the last diet of 50%:30%. The results showed that no significant difference in growth was found when abalone were fed with those diets. This indicated that varying the ratio of carbohydrate sources has no affected on growth of abalone *Haliotis discus hannai*. In another study, Thongrod *et al.* (2003) determined the optimum ratio of carbohydrate versus lipid levels for growth of tropical abalone *H. asinia*. The abalone were fed with an isoenergetic base diet consisting of soybean and

Spirulina as protein sources at protein level of 37 %. Fish oil and modified starch were added to the base diet to obtain the different ratio consisting of 1.3:47.8 % (lipid/carbohydrate, v/v), the second was 5.8:43.4 %, the third one was 10.2:39.5 %, the fourth one was 14.7:36.1 % and the last one of 19.0:31.2 %. This study showed that there was a significant difference in weight gain of abalone between the tested diets (Table 5.1). The abalone that were fed diet containing higher levels of carbohydrate as an energy source performed well whereas lipid played a negative if any role as energy source. The higher lipid levels (>5.8 %) could probably not help abalone growth.

**Table 5.1.** Weight gain and feed conversion ratio of abalone fed various lipid/carbohydrate levels.

Dietary (lipid/carb.)	Weight gain (%)	FCR
1.3:47.8	777.9 <sup>a</sup>	1.31 <sup>a</sup>
5.8:43.4	614.7 <sup>b</sup>	1.39 <sup>a</sup>
10.2:39.5	353.9 <sup>c</sup>	1.90 <sup>ab</sup>
14.7:36.1	222.7 <sup>d</sup>	3.20 <sup>b</sup>
19.0:31.2	97.5 <sup>e</sup>	7.43 <sup>c</sup>

Value with different letters indicate significantly difference ( $P < 0.05$ ) (Thongrod *et al.*, 2003).

Utilization of various carbohydrate sources were tested on growth performances of abalone *H. discus hannai* (Lee *et al.*, 1998). Juvenile abalone were fed with various diets containing 24.2 % wheat flour, 20 % dextrin, 20 % sucrose, 10 %  $\alpha$ -potato starch + 10 %  $\beta$ -potato starch, 15 %  $\alpha$ -potato starch, 20 %  $\alpha$ -potato starch, and 25 %  $\alpha$ -potato starch. A control diet was a mixture of soybean meal, corn gluten meal, and wheat flour. All these diets were compared to the control, which abalone were fed with natural diet of *Laminaria* and *Undaria*. The results showed that weight gain of abalone was not significant due to different carbohydrate sources in diet (Table 5.2), except it was significantly higher compared to natural feeds *Laminaria* and *Undaria*. The authors thought the difference in performance could probably due to lack of essential amino acids in natural feeds. Cho *et al.* (2008) reported that *Laminaria* has a poorly balanced amino

acid content. Other authors successfully fed carbohydrate to abalone but did not systematically test them (Cho *et al.*, 2008; Cho 2010).

**Table 5.2.** Weight gain of abalone fed various carbohydrate sources.

Carbohydrate sources	Weight gain (%)
Wheat flour	224 <sup>cd</sup>
Dextrin	228 <sup>cd</sup>
Sucrose	249 <sup>d</sup>
10% $\alpha$ -potato starch + 10% $\beta$ -potato starch	250 <sup>d</sup>
15% $\alpha$ -potato starch	196 <sup>bc</sup>
20% $\alpha$ -potato starch	229 <sup>cd</sup>
25% $\alpha$ -potato starch	229 <sup>cd</sup>
Mixed	228 <sup>cd</sup>
<i>Laminaria</i>	157 <sup>b</sup>
<i>Undaria</i>	111 <sup>a</sup>

Values with different letters indicate statistically significant differences ( $P < 0.05$ ) (Lee *et al.*, 1998).

In the study to be described, a systematically varied carbohydrate level was tested to determine the optimal carbohydrate level on growth performance of opihi. We believe that this is the first study to have attempted to examine the growth response of opihi, among the gastropod species to various carbohydrate levels, while maintaining constant protein and lipid levels in the diet.

## MATERIALS AND METHODS

Experimental diets. Four different dietary carbohydrate levels were formulated as Table 5.3. A base diet of 27 % protein and low lipid level of 3.5% were used. Wheat flour was used as carbohydrate source and diatomaceous earth was used to balance in the diets as carbohydrate level was decreased. Protocols for diet preparation, feeding regime and measurement of feed

consumption rate are described in previous chapter and Hua and Ako (2012). Typically 9g of dry ingredients and 9g of water (not shown) were used in a 1:1 ratio.

**Table 5.3.** Composition of dietary carbohydrate level.

Ingredient (g/100)	Dietary carbohydrate level (%)			
	18	27	32	37
Fishmeal	16.5	16.5	16.5	16.5
Soy meal	11.5	11.5	11.5	11.5
Krill meal	4.50	4.50	4.50	4.50
Porphyra *	14.0	14.0	14.0	14.0
Wheat flour	15.4	26.9	33.7	40.5
Diatomacetous earth	30.9	19.4	12.6	5.80
Alginate	5.00	5.00	5.00	5.00
Corn/fish oil **	1.00	1.00	1.00	1.00
Vit. mix ***	1.00	1.00	1.00	1.00
Cholesterol	0.20	0.20	0.20	0.20
Total	100	100	100	100
Calculated nutrient				
Protein	26.5	26.5	26.5	26.5
Lipid	3.47	3.47	3.47	3.47

\*This is commercial seasoned seaweed known as nori or the red algae

*Porphyra tenera* or *yezoensis*. Nishimoto Trading Co. Ltd., Korea

\*\* Mixture of corn oil and menhaden oil (1:1; v/v)

\*\*\* Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute).

Gelatinization. In this study the process of gelatinization was conducted. A volume of 9 mL of water was added into a beaker (25 mL) containing wheat flour and alginate. The beaker was then placed into the boiling water beaker (250 mL) on a hot plate and stirred while heating. The gelatinization occurred when water temperature increased in a few minutes; the solution became more and more viscous and turned into a thick gel. Other ingredients were then mixed thoroughly with the gelatinized solution and thereafter the mixed was heated in boiled water bath

again for about 2 minutes. The paste was shaped into sheets about 1.0 mm thickness, and then cut into 1.0 cm<sup>2</sup>/pieces. The pieces were then sealed in a plastic sample bag and stored at -20°C until use.

Experimental animals. Two batches of collected opihi with mean shell length of 2.8±0.5 cm were used for this study. Nine individuals were tested with each diet and were stocked one group at a time. Each individual of each collection was measured to obtain their initial shell length and weight. The first animal was stocked into group one, the second animal was also stocked in group one, and so forth until all groups were populated. An alternative would be used to have stocked the first animal in group one, the second animal in group two, and so on so that there would be no systematic bias in stocking. Each opihi was placed in a soft colander (20 cm diameter). It was repeated for the animals of the second collection. This selection technique led to a biased stocking and the growth data of individual in each diet and all diets had to be normalized with hope of solving the error.

Colanders were distributed into large tubs (150 L) with a recycled water flow rate (3 L min<sup>-1</sup>). The experiment was conducted for 90 days, at water temperatures ranged from 23°C to 26°C. The growth of animals in weight (g) and shell length (cm) were measured monthly. The growth was expressed in terms of specific growth rate (SGR), percent weight gain and shell length increasing. The shell length were measured with a dial caliper (0.01 inch) and weight determined with an electronic scale (0.01 g accuracy) every month.

Due to a stocking error, the weight gain of each animal in each treatment was also normalized and being used for statistical analysis.

Normalized weight gain = (final weight-initial weight)/initial weight

SGR = ((lnWf-lnWi)/T) x 100, where Wf is final weight, Wi is initial weight and T is total day conducting experiment.

Feed consumption (% dry matter/ soft body weight/day) for individual = [feed eaten (g, dry weight)/ soft body weight (g)] x 100

Protein intake = total feed intake (g/individual) x (g protein/g dry feed)

Sampling and analytical methods. The growth of animals in weight (g) and shell length (cm) were measured monthly. The growth was expressed in terms of specific growth rate (SGR), weight gain and shell length increase. The shell length was measured with a dial caliper (0.01 inch) and weight determined with an electronic scale (0.01 g error) every 4 weeks

Mean weigh gain (g/individual) =  $W_f - W_i$

Weight gain (WG, %) =  $[(W_f (g) - W_i (g)) / W_i (g)] \times 100$

Specific growth rate (SGR) =  $\{(\ln W_f - \ln W_i) / T\} \times 100$ ,

where  $W_f$  is final weight,  $W_i$  is initial weight and  $T$  is total day of the experiment.

Feed conversion ratio (FCR) = total feed eaten (g)/wet weight gain (g).

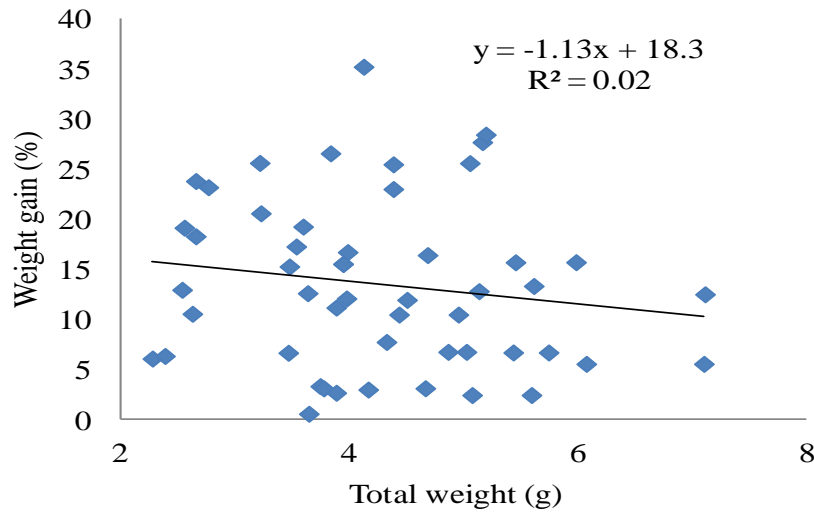
Water stability test. Feed water stability was conducted in experimental test system. Two representative dietary formulas of 18 % and 32 % carbohydrate levels were selected for this test, three replications per diet. The experimental procedures were conducted as previous description in chapter 4.

Statistical analysis. All feed consumption of animals was calculated and averaged by Excel. One way ANOVA followed by Tukey Kramer was used to compare mean feed consumption rate (%DM/BW/day), normalized weight gain (g), specific growth rate (SGR, %BW/day) among the trials.

## RESULTS

Gelatinization is a process of denaturing the starch chain that allows water absorption and complete destruction of the crystalline structure of the starch granule in the presence of heat and moisture. Gelatinization is important because it changes the taste and texture of a starch granule and makes it easier to digest. Our results show that under heat in water bath for a few minutes (about 5 min) wheat flour and alginate underwent gelatinization. The feed tasted like cooked rice.

A systematic error in initial stock size of animal occurred with the smaller size group was distributed to 18% carbohydrate diet, the next smallest animals in the 27 % groups and so on. Statistical analysis was done to determine if there was any relationship between different sizes and percent weight gain in these trials. The total weights of the all animals in all trials were plotted against their weight gain (%) for every measurement taken. Figure 5.1 shows the relationship between animals sizes and percent weight gain of animal. The relative equation is  $y = -1.13x + 18.3$ , at the  $R^2$  value of 0.024 which is nearly zero where a value of 1 would be a perfect correlation. Further regression analysis showed that there was no significant correlation ( $P = 0.27$ ) between the animal sizes and percentage weight gain. Thus, the data suggest that an error in initial stocking sizes did not affect the growth of animal in this study.



**Figure 5.1.** Scatter plot of weight gain versus the total weight of animals in carbohydrate diets. The regression line of best fit the relationship between the two factors and R values indicates the strength of the relationship.

The first month of data was straightforward in all trials. Due to stocking errors, the weight gain of each animal was normalized for all treatments. Animals that died were removed from calculations for and subsequent measurements and data was not recorded for them. Over time it was noticed that the partly mortalities occurred when animals were removed off the colander for weighing. The empty grids in the following tables represent dead animals.

Growth performances and feed consumption of opihi that were fed with dietary of 18 % carbohydrate level are shown in the Table 5.4. A mediocre survival rate of 67 % was obtained in this experiment after 3 months. Of the survivors, the normalized average weight gain was  $0.09 \pm 0.07$ g per 1g initial weight for the first month and  $0.15 \pm 0.05$  g and  $0.19 \pm 0.04$  g for the second and the third month measurements, respectively. The average specific growth rate was  $0.27 \pm 0.23$  %, and the variance among individuals in the first measurement ranging from 0.01 to 0.62%. Individual feed consumption rates also varied among the individuals. The average feed consumption rate was decreased from the first month of  $0.75 \pm 0.28$ %DM/BW/day to  $0.58 \pm 0.21$ %DM/BW/day and  $0.58 \pm 0.17$ %DM/BW/day for the second and third months respectively. FCR ranged from 0.63 to 1.11.

**Table 5.4.** Weight (g), SGR (%BW/day), feed consumption (%DM/BW/day) measurement of animal fed dietary of 18% carbohydrate.

	Animal No.									Mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	2.14	3.62	2.24	2.67	3.41	2.37	3.01	3.01	2.73	2.80±0.51
1 <sup>st</sup> month weight (g)	2.27	3.64	2.53	3.22	3.94	2.38	3.47	3.22	2.75	3.05±0.59
Normalized weight gain (g opihi <sup>-1</sup> )	0.06	0.01	0.13	0.21	0.16	0.06	0.15	0.07	0.01	0.09±0.07
SGR (%BW/day)	0.20	0.02	0.41	0.62	0.48	0.01	0.47	0.22	0.02	0.27±0.23
Feed consump.	1.09	0.85	1.12	0.78	0.99	0.57	0.41	0.42	0.49	0.75±0.28
2 <sup>nd</sup> month weight (g)	2.55	3.74	2.65		3.98	2.62	3.53	3.50	3.21	3.22±0.56
Normalized weight gain (g opihi <sup>-1</sup> )	0.19	0.03	0.18		0.17	0.11	0.17	0.18	0.16	0.15±0.05
SGR (%BW/day)	0.29	0.05	0.28		0.26	0.17	0.27	0.25	0.27	0.23±0.08
Feed consump.	0.83	0.59	0.93		0.56	0.46	0.36	0.50	0.38	0.58±0.21
3 <sup>rd</sup> month weight (g)	2.65		2.76		3.94		3.59	3.58	3.16	3.28±0.51
Normalized weight gain (g opihi <sup>-1</sup> )	0.24		0.23		0.16		0.19	0.19	0.16	0.19±0.04
SGR (%BW/day)	0.25		0.24		0.17		0.20	0.20	0.17	0.20±0.03
Feed consump.	0.75		0.84		0.52		0.46	0.46	0.44	0.58±0.17

A higher survival (78 %) was obtained in the second diet of 27 % carbohydrate (Table 5.5). The average normalized weight gain of opihi increased gradually after three months. Specific growth rate of animals at this diet was relatively increased from 0.12%BW/day to 0.27%Bw/day in the third month measurement. The average feed consumption rate was slightly decreased from the first month of 0.80±0.30%DM/BW/day to 0.59±0.18%DM/BW/day and 0.57±0.11%DM/BW/day for the second and third measurements respectively. The average FCR was 0.50±0.30.

**Table 5.5.** Weight (g), SGR (%BW/day), feed consumption (%DM/BW/day) measurement of animal fed dietary of 27% carbohydrate.

	Animal No.									Mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	3.56	3.49	3.46	3.78	4.02	3.12	4.04	3.13	3.54	3.57±0.33
1 <sup>st</sup> month weight (g)	3.67	3.63	3.69	3.88	4.5	3.21	4.16	3.40	3.84	3.78±0.39
Normalized weight gain (g opihi <sup>-1</sup> )	0.03	0.13	0.07	0.03	0.12	0.26	0.03	0.09	0.08	0.09±0.07
SGR (%BW/day)	0.10	0.13	0.21	0.09	0.38	0.09	0.10	0.28	0.27	0.18±0.11
Feed consump.	1.14	1.12	1.13	0.65	0.69	1.02	0.40	0.61	0.48	0.80±0.30
2 <sup>nd</sup> month weight (g)	3.97	3.88			4.68	3.85	5.16	3.90	4.15	4.23±0.50
Normalized weight gain (g opihi <sup>-1</sup> )	0.12	0.11			0.16	0.23	0.28	0.25	0.17	0.19±0.07
SGR (%BW/day)	0.18	0.18			0.25	0.35	0.41	0.37	0.26	0.29±0.09
Feed consump.	0.81	0.78			0.56	0.7	0.39	0.48	0.38	0.59±0.18
3 <sup>rd</sup> month weight (g)	4.38	4.38			5.05	4.12	5.19	4.01	4.25	4.48±0.42
Normalized weight gain (g opihi <sup>-1</sup> )	0.23	0.26			0.26	0.32	0.28	0.28	0.20	0.34±0.04
SGR (%BW/day)	0.24	0.26			0.26	0.32	0.29	0.28	0.21	0.27±0.07
Feed consump.	0.71	0.68			0.48	0.59	0.46	0.56	0.50	0.57±0.09

Table 5.6 shows growth performances of opihi that were fed with dietary 32 % carbohydrate level for three months period. Survival rate of 78 % was obtained in this experiment. The normalized weight gain increased rapidly after the first month measurement (0.05g) to the third month (0.27g). The average specific growth rate was varied among individual in the first measurement ranging from -0.05 to 0.54 %. This could probably due to poor adjustment from one to another feed and relative feed consumption rate was also varied among the individual. The normalized weight gain and SGR were then tracked to each other after three month periods. The average feed consumption rate was relative increased from the first month (0.40±0.12%DM/BW/day) to the third month of 0.60±0.27%DM/BW/day.

**Table 5.6.** Weight (g), SGR (%BW/day), feed consumption (%DM/BW/day) of animal fed dietary of 32% carbohydrate.

	Animal No.									mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	4.38	4.38	4.32	2.76	5.71	3.12	3.29	5.61	4.19	4.19±0.97
1 <sup>st</sup> month weight (g)	4.41	4.42	4.25	2.78	5.96	3.16	3.87	6.19	4.24	4.36±1.12
Normalized weight gain (g opihi <sup>-1</sup> )	0.01	0.01		0.01	0.04	0.01	0.18	0.10	0.01	0.05±0.06
SGR (%BW/day)	0.02	0.03	-0.05	0.02	0.14	0.04	0.54	0.33	0.04	0.12±0.19
Feed consump.	0.36	0.5	0.23	0.55		0.56	0.32	0.32	0.38	0.40±0.12
2 <sup>nd</sup> month weight (g)	4.95	4.57	4.39	2.98		4.16	3.87	6.49	5.62	4.63±1.08
Normalized weight gain (g opihi <sup>-1</sup> )	0.13	0.04	0.02	0.08		0.33	0.18	0.16	0.34	0.16±0.12
SGR (%BW/day)	0.20	0.07	0.03	0.13		0.48	0.27	0.24	0.49	0.24±0.17
Feed consump.	0.57	0.43	0.24	0.63		0.56	0.50	0.46	0.36	0.47±0.13
3 <sup>rd</sup> month weight (g)	5.44	5.19		3.15		4.15	4.35	6.69	5.94	5.03±1.26
Normalized weight gain (g opihi <sup>-1</sup> )	0.24	0.18		0.14		0.33	0.32	0.24	0.42	0.28±0.10
SGR (%BW/day)	0.25	0.20		0.15		0.33	0.32	0.25	0.40	0.25±0.07
Feed consump.	0.47	0.92		1.05		0.52	0.48	0.40	0.36	0.60±0.27

Table 5.7 shows the growth performances and feed consumption of opihi that were fed with dietary of 37 % carbohydrate. The survival rate was 67 %. The average normalized weight gain was fairly increased from the first month (0.06±0.05 g) to the second (0.16±0.06 g) and remained flat for third month (0.15±0.04 g). The SGR was varied among the individual, ranging from 0.13 to 0.49% in the first month. Some animals did not adjust well to this feed. The average feed consumption rate was decreased from the first month of 0.76±0.31%DM/BW/day

to  $0.48 \pm 0.11\%$  DM/BW/day and  $0.45 \pm 0.07\%$  DM/BW/day for the second and third measurements respectively. The mean FCR was ranged from 0.65 to 2.29.

**Table 5.7.** Weight, SGR, feed consumption (%DM/BW/day) measurement of animal fed dietary of 37 % carbohydrate.

	Animal No.									Mean±SD
	1	2	3	4	5	6	7	8	9	
Initial weight (g)	4.01	4.45	4.95	4.52	4.71	5.09	6.32	3.36	2.97	4.49±0.99
1 <sup>st</sup> month weight (g)	4.43	4.86	5.07	4.66	5.45	5.43	6.07	3.57	3.07	4.73±0.94
Normalized weight gain (g opihi <sup>-1</sup> )	0.10	0.07	0.02	0.03	0.16	0.07	-0.04	0.06	0.03	0.06±0.05
SGR (%BW/day)	0.33	0.29	0.08	0.10	0.49	0.22	0.44	0.20	0.11	0.25±0.15
Feed consump.	1.15	1.01	0.85	1.10	0.91	0.50	0.49	0.47	0.32	0.76±0.31
2 <sup>nd</sup> month weight (g)	4.95	5.02	5.59		5.98	5.74	7.10	3.75	3.42	5.19±1.20
Normalized weight gain (g opihi <sup>-1</sup> )	0.23	0.13	0.13		0.27	0.13	0.12	0.12	0.15	0.16±0.06
SGR (%BW/day)	0.35	0.20	0.20		0.40	0.20	0.48	0.18	0.24	0.28±0.11
Feed consump.	0.65	0.60	0.43		0.42	0.42	0.37	0.36	0.56	0.48±0.11
3 <sup>rd</sup> month weight (g)	4.32	5.13	5.61				7.11	3.96	3.72	4.98±1.27
Normalized weight gain (g opihi <sup>-1</sup> )	0.08	0.15	0.13				0.13	0.18	0.19	0.20±0.06
SGR (%BW/day)	0.09	0.16	0.14				0.14	0.19	0.20	0.15±0.04
Feed consump.	0.55	0.52	0.40				0.37	0.42	0.41	0.45±0.07

Growth performances of opihi that were fed at various carbohydrate levels are summarized in the Table 5.8. Overall, the animals performed poorly in all trials for the first month measurement; the average normalized weight gain was about 0.05g-0.09 g per g initial weight of opihi. Statistical analysis showed that there was no significant ( $P>0.05$ ) difference in normalized weight gain and the specific growth rates of those animals that were fed with various carbohydrate levels in the first month. The opihi gained about double weight in the second measurement, but statistical analysis showed that there was no significant difference in

normalized weight gain and SGR of animals. After a three month period, statistical analysis by One-way ANOVA showed that there was a significant ( $P < 0.05$ ) difference in weight gain and the specific growth rates of opihi among the diets, then a Tukey test showed that the weight gain of animals on the 18 % and 37 %diets was significantly ( $P < 0.05$ ) lower than animals fed 27 % and 32 % carbohydrate. However, there was no significant difference in specific growth rate of those animals that were fed with diets between the 18 % and 27 % and 32 % carbohydrate diets using  $P < 0.05$  criterion. The mean FCR values show that opihi are efficient converters of feed to body weight. Survival was about 90% to 100% for all diets after two months, however, the survival rates were from 67% to 78% after three months. The mortality was due handling stress from the second weighing, when we removed them from the colander walls.

**Table 5.8.** The growth performance of opihi fed various dietary carbohydrate levels; SGR, specific growth rate; feed consumption rate (%DM/BW/day).

Measurement	Dietary carbohydrate level (%)			
	18	27	32	37
Mean initial weight of survivors (g)	2.80±0.51	3.56±0.37	3.96±0.98	4.34±1.20
Mean 1 <sup>st</sup> month measurement (g)	3.05±0.59	3.78±0.39	4.36±1.12	4.73±0.94
Mean normalized weight gain (g opihi <sup>-1</sup> )	0.09±0.07 <sup>a</sup>	0.09±0.07 <sup>a</sup>	0.05±0.06 <sup>a</sup>	0.07±0.04 <sup>a</sup>
Mean SGR (%BWday <sup>-1</sup> )	0.27±0.23 <sup>a</sup>	0.18±0.11 <sup>a</sup>	0.12±0.19 <sup>a</sup>	0.19±0.18 <sup>a</sup>
Feed consumption	0.75±0.28 <sup>a,b</sup>	0.80±0.30 <sup>a</sup>	0.40±0.12 <sup>b</sup>	0.76±0.31 <sup>a,b</sup>
Mean 2 <sup>nd</sup> month measurement (g)	3.22±0.56	4.23±0.50	4.63±1.08	5.19±1.20
Mean normalized weight gain (g opihi <sup>-1</sup> )	0.15±0.05 <sup>a</sup>	0.19±0.07 <sup>a</sup>	0.14±0.13 <sup>a</sup>	0.19±0.08 <sup>a</sup>
Mean SGR (%BWday <sup>-1</sup> )	0.23±0.08 <sup>a</sup>	0.29±0.09 <sup>a</sup>	0.24±0.17 <sup>a</sup>	0.25±0.08 <sup>a</sup>
Feed consumption	0.58±0.21 <sup>a</sup>	0.59±0.18 <sup>a</sup>	0.47±0.13 <sup>a</sup>	0.48±0.11 <sup>a</sup>
Mean 3 <sup>rd</sup> month measurement (g)	3.28±0.51	4.48±0.42	5.03±1.26	4.94±1.31
Weight gain (g opihi <sup>-1</sup> )	0.52±0.05 <sup>b</sup>	1.13±0.15 <sup>a</sup>	1.06±0.42 <sup>a</sup>	0.60±0.16 <sup>b</sup>
Mean normalized weight gain (g opihi <sup>-1</sup> )	0.19±0.04 <sup>a,b</sup>	0.26±0.04 <sup>a</sup>	0.27±0.09 <sup>a</sup>	0.18±0.09 <sup>b</sup>
Mean SGR (%BWday <sup>-1</sup> )	0.20±0.03 <sup>a,b</sup>	0.27±0.04 <sup>a</sup>	0.26±0.07 <sup>a</sup>	0.15±0.04 <sup>b</sup>
Feed consumption	0.58±0.17 <sup>a</sup>	0.57±0.09 <sup>a</sup>	0.60±0.27 <sup>a</sup>	0.45±0.07 <sup>a</sup>
FCR	0.83±0.21	0.50±0.30	0.77±0.47	1.16±0.66
Survival (%)	67	78	78	67

<sup>a,b</sup> Means, within the row, different superscript letters are significant differently ( $P < 0.05$ )

The  $P < 0.05$  criterion is essentially universal in aquaculture. It means that if two means have a  $P < 0.05$ , they have only a 5 % probability of being similar. It might be instructive to take a further look at the Tukey Kramer analysis on normalized weight gain between animals fed 18 %, 27 %, 32 % and 37 % carbohydrate (Table 5.9). The animals fed the animals fed 37 % carbohydrate have a 0.001 % and a 0.003 % chance of being identical in growth rate to animals

fed the 27 % and 32 % diets. This makes it easy to decide what diet to choose. The animals 18 % carbohydrate diet have an 18 % or 15 % chance of being different from animals eating the 27 % and 32 % carbohydrate diets respectively. The  $P < 0.05$  criterion suggests that the 18% performs in a way that is not significantly different 27 % and 32 %. The Tukey Kramer number raises doubts as to the similarities and adds more definition. This will be discussed in greater detail later.

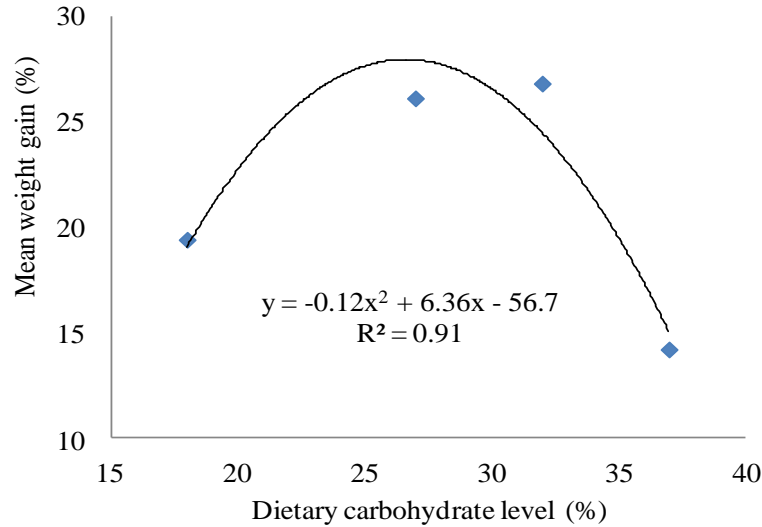
**Table 5.9.** The Tukey Kramer analysis between the treatments at 95% confidence interval.

Between diets (%)		Mean Difference	Std. Error	Significant at P value
18	27	-0.07	0.03	0.18
	32	-0.07	0.03	0.15
	37	0.05	0.03	0.50
27	18	0.07	0.03	0.18
	32	-0.003	0.03	1.00
	37	0.12*	0.03	0.009
32	18	0.07	0.03	0.16
	27	0.003	0.03	1.00
	37	0.12*	0.03	0.007
37	18	-0.05	0.03	0.50
	27	-0.12*	0.03	0.009
	32	-0.12*	0.03	0.007

\*the mean difference is significant different at the 0.05 level

Response of opihi in weight gain to dietary carbohydrate levels was then fitted to quadratic models (Fig. 5.2). It shows that the weight gains of opihi progressively increased and reached to maximum value at a level of about 28 %, which could probably be described as  $Y = -0.12x^2 + 6.36x - 56.7$  with the correlation value of  $R^2 = 0.91$ . However, the polynomial analysis is a non-

statistical method and should not be used in decision making because it does not involve calculations of probability.



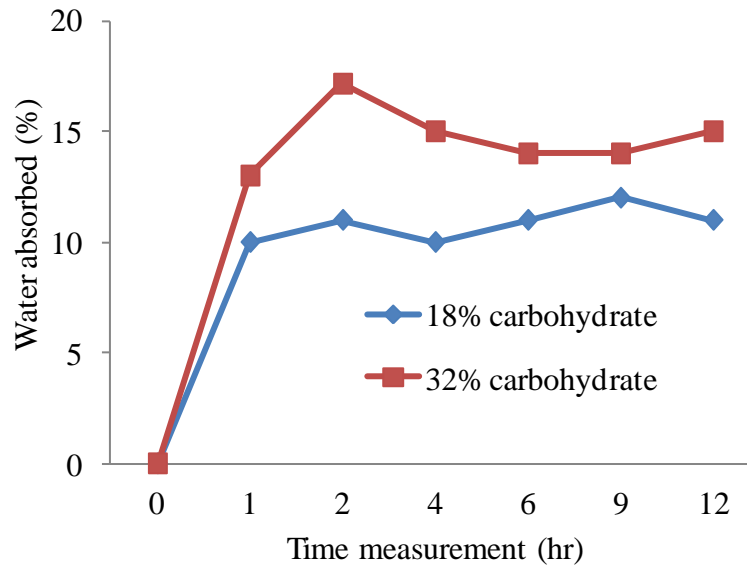
**Figure 5.2.** Relationship between normalized weight gain and dietary carbohydrate level for opihi as described by polynomial regression

Percentage dry weight retained of dietary 32 % carbohydrate was higher as compared to dietary 18% carbohydrate but there was no difference statistically in water stability of test diets after they were immersed for 12 hr (Table 5.10). Figure 5.3 showed that water was absorbed after the first hour then the feed weight was stable for the next 12 hr. Slightly different in the initial weight was due to difference in initial weight of pieces test.

**Table 5.10.** Water stability test of two dietary carbohydrate levels.

	Dietary carbohydrate level (%)	
	18	32
Mean wet weight (g)	0.326±0.007	0.347±0.002
Mean dry weight (g)	0.168±0.004	0.167±0.001
Mean dry weight after 12 hr (g)	0.160±0.005	0.165±0.001
Dry weight retained after 12 hr (%)	95.2±1.90 <sup>a</sup>	98.3±0.20 <sup>a</sup>

The data were presented as mean ± SD, n = 3; Means values within the row, Sharing the same superscript letters are not significant difference ( $P > 0.05$ ).



**Figure 5.3.** Feed wet weight immersing in seawater by the time.

## DISCUSSION

The purpose of this chapter was to determine the optimal dietary carbohydrate requirement for optimal performance of the opihi. The high mortality could raise questions about the strength of our data. We were originally thinking that mortality would be minimal and would occur randomly during the experiment. At this stage, believed we had all the handling and nutritional

variables taken care of and there should have been only few mortalities. However, in these longer term experiments we discovered handling mortalities when using colanders. Overall, there were 17 % out of 28 % total mortalities occurred after the animals were removed from colander walls for weighing. We had not expected this occur because our previous observation found that animals were easily removed off of colander at night while they moving or searching for food. The mortality in this study suggests that colander is not as good a holding facility for handling animal. Another kind of soft container was designed to overcome the colander problems. It was a cone made of soft polyethylene plastic as mentioned in Chapter 4.

A systematic error in the distribution of experimental animal's size among the treatments could have affected the results of this study. We made a mistake in distributing animals among experimental groups. Therefore, the weight gain of each individual had to be normalized in the hope of solving the issue. The regression analysis proved that there was no significant ( $P > 0.05$ ) difference between the initial sizes and the percentage weight gain of opihi even before normalization suggesting we had two methods to show that there was no consequence to stocking errors. The difference in the initial sizes did not affect the growth of opihi in the present study. Tung and Shiau (1993) reported that there was no significant difference in weight gain between the larger and smaller group of tilapia *Oreochromis niloticus* x *O. aureus* when they were offered starch diets. For abalone, a review by Fleming et al (1996) showed that a similar growth rate of abalone within the shell length ranging from 25-50 cm when they were offered the same diet. In our present study, a similar in initial shell length (2.80 - 3.11 cm, shell length) of animals was used.

We considered studies on carbohydrate requirement of abalone as a good indicator for the use of carbohydrate sources. Uki and Watanabe (1989) also reported that abalone could utilize dextrin as carbohydrate source. Later Lee *et al.* (1998) showed that abalone were able to digest either singlets or mixtures of dextrin, wheat flour, sucrose and potato starch. Thongrod *et al.* (2003) used modified starch as carbohydrate source. Wheat flour and alginate were used in abalone diet (Cho *et al.*, 2008; Cho 2010). These results suggest that the abalone would be able to utilize various carbohydrate sources in their diet but provide no quantitative comparisons.

The ability of fish to digest carbohydrate sources varies among the species and different carbohydrate levels as well (Dabrowski and Guderly 2002). An early work on the effect of various carbohydrate levels ranged from 0-48 % on growth of Chinook salmon *Oncorhynchus*

*tshawytscha* by using dextrin was reported by Buhler and Halver (1961). The weight gain of fish was progressively increased up to 20 % and remained almost the same when they were fed with dietary dextrin ranging from 30-48 %. This suggests that Chinook salmon have a limited capability for dextrin digestion in their diets when polysaccharide exceeds 20 %. In the next experiment, various carbohydrate sources were fed to Chinook salmon at level of 20 %. They found that glucose, maltose resulted in the best growth rates, followed by dextrin and the potato starch was the worst. This suggests that the Chinook salmon are lacking in high levels of polysaccharides digestive enzymes and that monosaccharides are immediately absorbed and used.

However, Channel catfish *Ictalurus punctatus* can utilize carbohydrate well up to 33% in their diets (Wilson and Poe 1987). In these experiments dextrin and cornstarch diets produced higher significant weight gains compared to glucose, maltose, fructose and sucrose, suggesting that polysaccharides are more effectively utilized than mono or disaccharides. Plasma glucose levels of fish that were fed dextrin diet were moderately increased compared to those of fish fed glucose or maltose. This indicates that dextrin had a slower digestion rate and was more effectively utilized than the rapidly absorbed glucose, maltose, fructose and sucrose diets. A quick absorption of glucose and prolonged high blood glucose could cause the growth depression. The same experimental design was tried for tilapia *O. niloticus* x *O. aureus*, when fish were tested on isonitrogenous diets containing 44% cornstarch, maltose, sucrose, lactose, glucose (Shiau and Chuang 1995). The authors found that a significant higher weight gain was obtained when fish fed with starch diets as compare to others. These data suggest that tilapia utilized polysaccharides more effectively than disaccharides or mono-saccharides. This may be explained that the presence of higher level of  $\alpha$ -amylase and  $\alpha$ -glucosidase in the digestive tract of these species. For example, in the whole digestive tract of common carp (*Cyprinus carpio*) contains higher level of  $\alpha$ -amylase (approximately 70 times) than that of gilthead sea bream (Hidalgo *et al.*, 1999). This showed that a high capability of polysaccharide utilization by the herbivores.

The results of our present study demonstrated that various carbohydrate levels in the diet affected growth performances of opihi. Statistical analysis showed that there was a significant ( $P < 0.05$ ) difference in both weight gain and SGR of opihi between the diets (Table 5.8). There was no significant difference between the weight gain of animal that fed with 27 % and 32 %

diets which indicates that the opihi could satisfy their needed for carbohydrate with either of the two diets. Although, there was no significant difference (using a  $P < 0.05$ ) in the average specific growth rate of animals fed with 18 % carbohydrate compared to those animals that were fed with 27 % and 32 % carbohydrate levels. However, further analysis the statistical results showed that the 18% diet had a tendency toward lower growth rates when compared to 27 % ( $P=0.18$ ) or 32 % ( $P=0.15$ ). Polynomial analysis suggests that 27 % carbohydrate diet would be an optimal level. On the other hand, the cost of feed would probably be higher at the lower carbohydrate levels. Carbohydrate is an inexpensive ingredient. It is noted that non-nutrient ingredients being used as a filler in diet are more expensive than carbohydrate sources. Therefore, the 32 % carbohydrate could be used as a final level for commercial opihi feed.

A lower carbohydrate level (18 %) may not be sufficient for adequate energy utilization by opihi and part of the energy needed may be catabolized from protein, therefore it may be reduce the optimal use of dietary protein for somatic growth. Study has shown that when there is an insufficient supply of carbohydrate in diet, it can be readily synthesized via the catabolism of proteins and lipids (Livingstone & de Zwaan 1983). It is unclear why a significantly lower growth rate was seen in animals fed 37 % carbohydrate, presumably because of the decrease in feed consumption.

Poor growth performance of opihi could be attributed to low feed consumption. We have found that feed consumption was correlated with the growth response of opihi (Hua and AKo 2012). A significantly lower growth rate of animals fed 37 % carbohydrate may be due to the lower level of feed intake (0.45%DM/BW/day). The lower feed consumption would be relatively decreased in carbohydrate utilization. Feeds with this high a carbohydrate level may have had a decreased palatability. Moreover, water stability test confirmed that no significant difference in retained dry weight of 18 % ( $95.2 \pm 1.9$  %) and 32 % ( $98.3 \pm 0.2$  %) after 12 hr submerging in sea water, suggesting that relatively no significant difference in dry matter leaching (less than 5 %) among the tested diets.

In conclusion, there was a significant effect of different levels of carbohydrate on growth performance of opihi in this study. The 32 % carbohydrate could be used as a final level for commercial opihi feed if there are no other factors of concern rather than the feed cost. This study is the first we are aware of that quantitative carbohydrate requirement for among aquatic organisms.

Overall, it may be concluded that we have investigated a wide set of important feed palatability and nutrient factors and we should be able to generate a commercial feed for the aquaculture of opihi from our conclusions. One possible flaw in the diets tested here was the inclusion of diatomaceous earth which served no purpose other than that to act as of a filler for the diets, and we believe it can be used in much smaller proportions. Diatomaceous earth is considered an expensive ingredient. In the previous chapter, a systematic test for feed palatability was conducted and eventually we found *Porphyra*, which is an important key attractant that induced a good palatability. Furthermore, we also found that addition of krill meal into the diet containing fish, soybean meal resulted in improved even better feed palatability and growth performance. The other optimal macronutrient required for optimal growth was protein. A level of 350% was determined to be the highest protein level at which produced the best growth in the previous chapter. In this study, carbohydrate inclusion was examined at the level of 27 % and 32 %. As stated above, the cost of feed would probably be higher at the lower carbohydrate levels. Carbohydrate is an inexpensive ingredient. Therefore the carbohydrate level will be 32%. Therefore a final formulated diet should have been generated as shown in the Table 5.11.

**Table 5.11.** Composition of formulated diet for commercial aquaculture opihi.

Ingredient	g/100g
Fishmeal	21.0
Soy meal	16.6
Krill meal	11.0
Porphyra *	14.0
Wheat flour	28.3
Diatomaceous earth	1.80
Alginate	5.00
Corn/fish oil **	0.90
Vit mix ***	1.00
Cholesterol	0.40
Total	100
Calculated nutrient	
Protein	35.8
Lipid	5.13
Carbohydrate	32.0

\*This is commercial seasoned seaweed known as nori or the red algae *Porphyra tenera* or *yezoensis*.  
Nishimoto Trading Co. Ltd., Korea

\*\* Mixture of corn oil and menhaden oil (1:1; v/v)

\*\*\* Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute)

## CHAPTER 6 . REPRODUCTIVE PERFORMANCE, FINAL MATURATION AND SPAWNING INDUCTION OF OPIHI

### INTRODUCTION

The purpose of this chapter is to investigate the spawning seasons, induction of final maturation, spawning, and larval rearing of the opihi. We have investigated and learned how to handle opihi with minimum mortality in an aquaculture system. We have developed a feasible commercial aquaculture feed for opihi. This final diet provided good palatability and good growth performance. The protein level of 35 % and the 32 % carbohydrate level minimized feed cost. For this chapter we want to understand reproduction of the opihi. This will be beneficial for the understanding of the completion of the life cycle of Hawaiian opihi and would make opihi aquaculture sustainable. The success of any aquaculture species depends on seed production in captivity. Our first aim is to investigate the spawning seasons of opihi. This would provide us with better knowledge for breeding opihi in the hatchery. We then will attempt to look at how we could induce final maturation in opihi in the laboratory using artificial diets and/or using gonadotropin releasing hormone. In the interim before the life cycle has been successfully closed, wild broodstock must be used for spawning trials.

The first objective is to determine the spawning season(s) of opihi. Corpuz (1983) reported that there were two spawning season peaks that occurred between March to May and October to December. In our study of the yellow foot opihi *C. sandwicensis*, however, we will find that the March to May spawning period does not appear to exist. We expanded Corpuz's study by monthly measurement of the gonadosomatic index (GSI) and histology of gonads over a whole year. The highest GSI values were obtained in November and January suggesting a winter spawning season for the yellow foot opihi.

Induction of final maturation is another area of study. Relying on wild opihi broodstock in a long term is unacceptable due to the danger in collecting wild animals and uncertainty as to whether collected broodstock are ripe or not (Corpuz 1981). Final maturation might involve the arachidonic acid (ARA) to eicosapentaenoic acid (EPA) ratio. Several studies have shown that an appropriate ARA/EPA level in diet would produce maturity in aquatic animals. Arachidonic acid serves as a precursor for prostaglandins which are functional for reproductive process (Schimz and Ecker 2008). Prostaglandins play a critical role during the ovulatory process in

teleost fishes (Goetz 1983). For example, Tamaru *et al.* (1992) found a significantly lower level of ARA in spawned eggs of mullet broodstock held indoors than the ARA content in spawned eggs of broodstock held outdoors. This was related to lower percentage of fertilized eggs of broodstock held indoors.

Several ARA/EPA ratios are summarized in Table 6.1. The high ARA/EPA ratio (0.69) observed by Tamaru *et al.* (1992) was associated with a significantly higher fertilization rates. This could be traced back to the broodstock diet. Both groups were fed the same artificial diet at equivalent rations, but the outdoors-reared broodstock had benthic diatoms available and an analysis of the contents of their stomach suggested that they ate the benthic diatoms. These results strongly pointed to the desirability of increasing the ARA content of mullet broodstock diet, which probably contributed to the final maturation processes and subsequent eggs quality. The ARA was from diatoms eaten by broodstock held outdoors. The diatoms could also be a seasonal trigger signaling spawning of mullet as they bloom just before the spawning season.

In another study, Tamaru *et al.* (1997) also demonstrated that ARA played an important role in reproductive processes of freshwater ornamental fish (Table 6.1). All of the maturation diets used by breeder contained a high level of 20:4n-6, but low levels of EPA resulted in very high ARA/EPA ratios that are beyond those seen in marine species. This may mean that marine and terrestrial animals have eicosanoid enzymes that function in different omega-6/omega-3 environments. Uki *et al.* (1986) demonstrated the ability to convert 18:2n-6 and 18:3n-3 to 20:4n-6 and 20:5n-3 in abalone, respectively, but the process of bioconversion occurred at low rates. A low bioconversion rate led to low level of 20:4n-6 and would result low levels of prostaglandins. Therefore, there may be a requirement for ARA for animals. No studies have been done in opihi, but it may be assumed that a similar metabolic process would probably occur in opihi.

Furuita *et al.* (2003) also found that addition of ARA in broodstock diet improved eggs and larval quality of Japanese flounder, *Paralichthys olivaceus* (Table 6.1). Three different ARA levels of 0.1%, 0.6% and 1.2% dry matter were added into formulated diet to obtain relative ARA/EPA ratio levels of 0.12, 0.70 and 1.49. Broodstock were fed with these diets for 3 months before and during the spawning season. The results showed that the fish fed with diet containing 0.70 ARA/EPA had significantly higher egg production and hatching rate than fish fed with other diets. Moreover, ARA to EPA ratio levels of spawned eggs were also proportional to

ARA/EPA level of diets. Therefore, this study revealed a needed dietary ARA/EPA ratio of 0.70 was an optimal ratio that would be sufficient for reproductive performance of Japanese flounder.

ARA to EPA ratio in benthic diatoms is considered as a good reference. Benthic diatoms are a final natural maturation feed for a number of species. Our previous study found that opihi preferred feed on benthic diatoms in the wild (Hua and Ako 2012). Several benthic diatoms such as *Nitzschia*, *Amphora*, and *Navicula* were predominant in stomach content of opihi. Literature studies on several diatoms for ARA and EPA are shown in the Table 6.1. The ARA/EPA ratios vary, ranging from 0.02 to 0.97 (Gordon et al., 2006; Ako 1995; Volkman *et al.*, 1989). An ARA/EPA ratio of about 0.70 was found in *Nitzschia*. Other benthic diatoms such as *Chaetoceros* species contain ARA/EPA ratios of 0.51 to 0.97, which are also close to 0.70, but a lower level in *Amphora*, *Navicula* and *Thalassiosira*.

We suspected that ARA/EPA in mature ovary of opihi would be also a good indicator to generate a good maturation diet. Our data analysis for fatty acids of mature ovary (based on high GSI) of wild opihi showed low ARA level of 0.08 g/100g of dry matter and an ARA/EPA ratio of 0.08. This ratio was low compared to the ratio from the above studies and the meaning of this is unclear.

**Table 6.1.** The ARA/EPA ratio from several trials on maturation.

Species	Diet	ARA (g/100 g DM)	EPA (g/100 g DM)	Ratio (ARA:EPA)	Reference
Mullet	Spawned egg held outdoors	4.00 <sup>b</sup>	5.80	0.69*	Tamaru <i>et al.</i> , 1992
	Spawned egg held indoors	2.50 <sup>a</sup>	4.70	0.53	
Fresh water ornamental fish	Beef heart	5.10	1.10	4.64	Tamaru <i>et al.</i> , 1997
	Beef liver	2.20	0.00	-	
	Black tubifex	9.00	6.10	1.47	
	Red tubifex	6.40	3.30	1.94	
	Daphnia	1.60	0.70	2.29	
	Earth worms	2.20	0.90	2.44	
	Mosquito	3.30	2.30	1.43	
Japanese flounder, <i>Paralichthys olivaceous</i>	Diet 1, 0.1% ARA	0.04	0.32	0.12	Furuita <i>et al.</i> , 2003
	Diet 2, 0.6% ARA	0.24	0.32	0.70*	
	Diet 3, 1.49% ARA	0.45	0.30	1.49	
Diatoms	Nitzschia	0.05	0.08	0.68*	Gordon <i>et al.</i> , 2006
	Amphora	0.04	0.13	0.27	
	Navicula	0.02	0.22	0.10	Ako 1995
	Chaetoceros calcitrans	5.70**	11.1**	0.51	Volkman <i>et al.</i> , 1989
	Chaetoceros gracilis	4.50**	4.60**	0.97	
	Thalassiosira	0.30**	19.3**	0.02	
Opihi	Mature ovary	0.08	0.96	0.08	Analyzed

<sup>a,b</sup>, different letters indicate significant difference in ARA level among the trials in the same study. \* indicates the best ARA/EPA ratio as compared to other levels (without \*) in the same column. \*\* values presented as percentage total fatty acid.

Another aspect in the controlling reproduction of animal is gonadotropin releasing hormone (GnRH). The GnRH is a neuropeptide hormone which plays an important role in controlling the reproduction among vertebrates and invertebrates. In vertebrates, GnRH is originally elicited from the hypothalamus, where it stimulates the release of luteinizing hormone-releasing hormone (LH-RH) and follicle stimulating hormone-releasing hormone (FSH-RH) from the anterior pituitary (Schally *et al.*, 1971a,b; Baba *et al.*, 1971; Matsuo *et al.*, 1971a,b). LH and FSH regulate gametogenesis and steroidogenesis. The process of GnRH regulating reproduction of vertebrates was grouped into the brain, hypothalamus-pituitary-gonad chain or HPG axis. In fish, the GnRH was found and regulated the reproduction cycles of many fish species (Sherwood *et al.*, 1983; Sherwood *et al.*, 1994) and the process of reproduction is also mediated by HPG axis. Hormonal manipulations for the induction of final maturation and spawning have become important applications for practical aquaculture, especially for fish that do not undergo final oocyte maturation and spawning in captivity. For example, the hormonal induction of final maturation and spawning of striped mullet *Mugil cephalus* were successful by LH-RHa (Lee *et al.*, 1987). The fish were induced to final stages of maturation and spawning by two injections of priming with either carp pituitary homogenate at 20-40 mg/g body weight or LH-RHa of 0.10 mg/g BW followed by a resolving dose of LH-RHa of 0.20-0.30 mg/g BW in 24 hr later. In another research on milkfish, *Chanos chanos*, Lee *et al.* (1986a,b) used LH-RH analog to induce the vitellogenesis processes and spawning by implantation or injection at average dose of 42 µg/kg body weight (BW) and 59 µg/kg BW respectively. The authors also stated that maturation and spawning of fish could be obtained at one, two or three injections but later spawns were natural because the timing of artificial hormonal induction of spawning had a window of opportunity of only a day and it was easier to let the fish spawn by themselves.

Several studies have showed that GnRH is also active in invertebrate species. GnRH-like peptides that existed in central nervous system and peripheral chemosensory organ of sea hare *Aplysia* were detectable by antisera against mGnRH (Tsai *et al.*, 2003). These GnRH-like peptides controlled egg laying of *Aplysia*. For abalone, studies had demonstrated the existence of GnRH-like peptides in the neural ganglia and ovary of the abalone (Nuurai *et al.*, 2010; Amano *et al.*, 2010). The existence of GnRH-like peptide in the neural ganglia was determined by using immunohistochemistry and reverse phase-high performance liquid chromatography (Amano *et al.*, 2010). The in vitro assay revealed that GnRH-like immunoreactive material

(reacted with antibody against mGnRH analogs) was detected in the peripheral region, neuropil of the pedal ganglion, the visceral nerve, and the nerve originating from the pedal ganglion. The cerebral ganglion contains A cells, which are responsible for the vitellogenesis and pleural-pedal and visceral ganglia are functional for the gametogenesis and induction of spawning in abalone (Hahn 1994). Nuurai *et al.* (2010) also revealed the presence of GnRH-IR in ovary of tropical abalone, suggesting that GnRH-like peptides are produced in the cerebral ganglion, then they are transported to pleural-pedal ganglion via the nerve and final destination of visceral which function for ovulation. They also reported that mammalian GnRH<sub>a</sub> and octopus GnRH<sub>a</sub> were affective in controlling the reproductive processes of abalone. They found that the adult abalone got final maturation in 5 weeks by weekly injection of these GnRHs at low dose (250 ng/g BW) and induced spawning at higher dose of 1,000 ng/g BW. These results suggest that the synchronous maturation and spawning of abalone caused by the GnRH stimulatory affect the neurosecretion system. It was reported that the neurosecretory cells were found abundantly in molluscan ganglia and they are the principal source of hormone that regulate the reproduction of abalone (Hahn 1994). These results suggest the same biological function in opihi.

In the study to be described in the present chapter, the induction of final maturation of opihi was approached using ARA supplementation in broodstock diet and injection of salmon GnRH analog. The final maturation process of opihi was observed when animals were fed with diet containing different ARA levels. The same ARA/EPA ratio of 0.7 was used as a base level. The opihi were also induced to final maturation using salmon GnRH analog at dose of 250 ng/g BW. The results will show that the gonads of opihi developed rapidly and reached to the maximum level after 4 weeks of injection as compared to the control. The classification of maturity stages of gonad development of opihi were characterized in the Table 6.2, which were described by other reports for limpets species (Corpuz 1983; McCarthy *et al.*, 2008).

**Table 6.2.** Maturation stages of gonad of limpets (Corpuz 1983; McCarthy *et al.*, 2008).

Stage	Description
1 Resting stage	The gonad is characterized by little or germinal epithelium, unclear distinguishable from ovary-wall cells and also for spermatid, the initial oocytes about 2 $\mu\text{m}$
2 Early development	Nucleus enlarged, oocyte diameter about 7-10 $\mu\text{m}$ . The male gonad is shaped like around tubule and a thick germinal epithelium lines the edges of the testes lobes.
3 Late development	The ovaries are swollen laterally and some oocytes in the final stages of vitellogenesis. Cytoplasm granular, the oocytes diameter ranging from 50-100 $\mu\text{m}$ .
4 Ripe	Ovaries are swollen. Oocytes diameter ranging from 110-130 $\mu\text{m}$ . The testis is densely with spermatozoa, dark blue stained by hematoxylin.
5 Spawning and reabsorbing	Spawning testis contained about 80% mature sperm, the ovary contains less densities of mature oocytes relative to ripe gonads.

In this chapter, we also dealt with spawning and larval rearing. In spite of the remarks about wild broodstock, in this study we used a short cut by working with naturally ripe broodstock for spawning trials while we are waiting for our broodstock to mature in the laboratory. We tried two different spawning methods to examine the optimal method of spawning. The first method was conducted using hydrogen peroxide. Hydrogen peroxide is a traditional method used for spawning induction in abalone. Addition of  $\text{H}_2\text{O}_2$  to seawater is believed to produce hydroperoxy free radicals ( $\text{HOO}^\cdot$ ), and peroxy radicals ( $\text{OO}^\cdot$ ), these radicals of activated oxygen suitable for the cyclooxygenase catalyzed addition of prostaglandin (Morse *et al.*, 1977; Morse 1984; Moss *et al.*, 1995). We also attempted to use  $\text{H}_2\text{O}_2$  to induce spawning in opihi. The results will show that  $\text{H}_2\text{O}_2$  was not a practical method and not recommended for spawning induction in opihi. This was due to a non-specific effect, and the broodstock eventually dying within a week after being exposed to  $\text{H}_2\text{O}_2$ . Whereas, the second method with GnRH at dose of 1,000 ng/g BW may be considered as the most practical induction spawning

method for opihi because there were no mortalities occurring after spawning. Embryonic and larval development stages of yellow opihi *C. sandwicensis* were observed along from fertilized eggs to the completed larval stage and ready for settlement.

Opihi larval rearing was another aim, and it was also a focus in this chapter. The role of benthic diatoms *Navicula*, *Amphora*, *Nitzschia* and others was reported to best diatoms species induced the settlement and metamorphosis of abalone larvae (Searcy-Bernal *et al.*, 1992; Gapasin and Polohan 2005; Gordon *et al.*, 2006). The effects of different benthic diatoms grown on different plate-substrates on metamorphosis of the tropical abalone *Haliotis asinina* were reported by Gapasin and Polohan (2005). There was a significantly higher metamorphosis rate of abalone larvae to diatoms slurry (contained mixture of diatoms *Amphora*, *Coscinodiscus*, *Coconesis*, *Nitzschia*, *Diploneis* and *Mastoglia*) than *Amphora*, *Amphora+Nitzschia*, and *Nitzschia* with any plate-substrate. This indicated that multiple benthic diatoms seemed to be superior to a single benthic diatom. On the other hand, the plate-substrates of roughened plexiglass, corrugated plastic sheet and the rubberized canvas seemed to be preferred for settlement over fibrocement board. Gordon *et al.* (2006) also found that a mixture of benthic diatoms consisting of *Navicula* and *Amphora* produced a significantly higher in growth and survival rates than monocultures benthic diatoms; *Navicula*, *Amphora*, *Nitzschia* and/or mixed *Amphora+Nitzschia* for abalone larvae *H. discus hannai*. The report showed that the monocultures of benthic diatoms produced a poor growth and did not support survival for more than two weeks especially *Nitzschia*. The authors also stated this could be due to difference in nutritional value of these benthic diatoms. In particular, the EPA value in *Navicula* and *Amphora* was reported to be higher than the value in *Nitzschia* (Gordon *et al.*, 2006).

An early attempting experienced on the settlement of opihi larvae on different substrata was conducted by Gladys Corpuz (1983). She found that mylar plastic and plexiglass induced a significantly higher larval settlement compared to glass, smooth and rough basalt rocks, coral skeleton, textured and untextured plastic. However, the settlement rates were very low ranging from 1.58 % to 7.73 %. It could probably due to inappropriate benthic diatoms because all the substrates were coated with a thin film. These substrata were exposed to running seawater for a month prior to use. The component of film was not specifically determined.

In this chapter, an attempt was made to observe the settlement, survival and metamorphosis of the opihi larvae on different diatoms and pelagic algae. The results will show that the settled larvae died after 10 days.

## **MATERIALS AND METHODS**

Spawning season determination. To investigate the spawning seasons of opihi, at least twelve adults opihi (greater than 2.5 cm, shell length) were collected monthly from November 2011 to December 2012. They were carefully dissected to obtain their gonad's weight and body weight (soft tissue). The GSI was calculated according to the equation  $GSI = (GW/BW) \times 100$ ; where GW is gonad weight and BW is body weight or soft body tissue. Several gonads were also used for histological examination for development stage. After dissecting, their gonads were immediately fixed in 10 % formalin for histological examination. Histological sections were about 5-7 $\mu$ m each and were stained with hematoxylin and eosin at the Pathology Laboratory, Queens Hospital, Honolulu, Hawaii. The sex ratio of apparent male and female opihi was successfully determined during the spawning season. It is noted that this success requires killing of the animals and the certainty is less when animals must kept alive.

Potential fecundity was defined as the total number of counted oocytes present in a mature ripe female opihi, and was examined for at least 2 opihi during the spawning season from October to December. The gonads were carefully extracted from the animals, then weighed to obtain the gonad weight. A sub-sample approximately 0.10 g was taken at different locations of each ovary; apical part, middle and near the base. Each subsample was then mixed with 5mL formalin (10 %). The numbers of oocytes were counted and total number of oocytes in the ovary was estimated and presented as potential fecundity.

Maturation trials. The initial maturity experiment was conducted with the formulated growout opihi diet (Table 6.3). This diet was determined to be a good diet that has a good palatability and produced good growth performance in Chapter 3. Eight to nine adults opihi (3.07 $\pm$ 0.22 cm in shell length) were fed with this diet for three months period (May 2012-August 2012) in a recirculating seawater system. Seawater was exchanged about 30% every week. The experiment was conducted under natural photoperiod and ambient temperature ranging from 23°C to 25°C. Salinity was maintained at 35 ‰. Prior to beginning the experiment, several animals were sacrificed to obtain initial GSI and gonad status. In order to assess the growth of opihi gonad, 2-3 animals were randomly sampled monthly. Their gonads

were extracted and weighed to obtain gonad's weight for the calculation of the GSI. The gonad development stage was also determined histologically. Maturity stages of gonad development were characterized as described in the Table 6.2.

**Table 6.3.** Composition of formulated diet used for initial mutation trial.

Ingredient	g/100g
Fishmeal	25.5
Soy meal	20.5
Krill meal	13.5
Porphyra*	14.0
Wheat flour	12.2
Diatomaceous earth	7.30
Alginate	5.00
Corn/fish oil**	0.60
Vitamin mix***	1.00
Cholesterol	0.40
Calculated nutrient	
Protein	42.4
Lipid	4.97
Carbohydrate	18.3

\*This is commercial seasoned seaweed known as nori or the red algae *Porphyra tenera* or *yezoensis*. Nishimoto Trading Co. Ltd., Korea.

\*\* Mixture of corn oil and menhaden oil (1:1; v/v)

\*\*\* Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute)

The fatty acid profile of the growout diet is presented in the Table 6.4 (from Z.Y. Ju, Oceanic Institute).

**Table 6.4.** Fatty acid composition of growout diet used for maturation trial.

Fatty acid	g/100 g
C14:0	0.47
C16:0	1.36
C16:1n-7	0.38
C18:0	0.20
C18:1n-9	0.91
C18:2n-6	1.22
C18:3n-3	0.11
C18:4n-3	0.07
C20:0	0.07
C20:1n-9	0.02
C20:4n-6	0.02
C20:5n-3	0.52
C22:5n-3	0.01
C22:6n-3	0.23
Total fatty acid	6.95*
ARA/EPA	0.04

\*includes minor fatty acids not listed

It will be shown later, the growout diet did not lead to the final maturation and other diets had to be formulated based on ARA/EPA ratio. The first diet served as a control without additional ARA. The second diet had a target level of ARA at a ARA/EPA ratio of 0.70. This ratio level was calculated as based on the composition of the growout diet (Table 6.3), fatty acid analysis data of the growout diet in the Table 6.4, and the EPA contents of the menhaden fish meal used and menhaden fish oil (NCR 2011). These were 0.895 g/100 g (Oceanic Institute data) and 11 g/100 g, respectively. The EPA level of the growout diet was 0.52 g/100g. These facts allow calculation of the EPA level of krill meal of 1.92 g/100g. The first reformulated diet served as a control diet (without addition of ARA) contained 16.5 g fishmeal and 4.50 g krill

meal and 0.10 g fish oil (Table 6.5). The second diet is the same as the first diet. Thus the EPA level in second reformulated diet “Low ARA” would be approximately 0.25 g, therefore an ARA should be 0.20 g to achieve desirable 0.70 ARA/EPA ratio. ARA was added in the form of pure arachidonic acid purchased from Sigma Chemical Co. The third diet is the same as the low ARA diet except fish oil was raised up to 2% to boost up EPA level to 0.47 g/100 g. It triggered increasing the ARA level in diet “High ARA” would be approximately 0.33 g/100 g. The theoretical levels of ARA and EPA are shown on the bottom of the table.

**Table 6.5.** Composition of formulated diets with different ARA levels.

Ingredient (g/100g)	Control	Low ARA	High ARA
Fishmeal	16.5	16.5	16.5
Soy meal	11.5	11.5	11.5
Krill meal	4.50	4.50	4.50
Porphyra*	14.0	14.0	14.0
Wheat flour	41.3	41.3	41.3
Diatomaceous earth	5.80	5.59	3.65
Alginate	5.00	5.00	5.00
Corn oil	0.10	0.10	-
Menhaden oil	0.10	0.10	2.00
Vit mix**	1.00	1.00	1.00
Cholesterol	0.20	0.20	0.20
ARA***	-	0.20	0.33
Total	100	100	100
Calculated nutrients			
Protein	26.5	26.5	26.5
Lipid	2.69	2.69	4.69
ARA	0.008	0.20	0.33
EPA	0.25	0.25	0.47
ARA/EPA	0.03	0.80	0.70

\*This is commercial seasoned seaweed known as nori or the red algae

*Porphyra tenera* or *yezoensis*. Nishimoto Trading Co. Ltd., Korea

\*\* Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute).

\*\*\* Purified ARA, PM Biomedicals, LLC; Fisher Science.

The fatty acid profiles of the control and the low ARA diets were obtained from Dr. Ju at Oceanic Institute for the confirmation of ARA level in the diets. The results of fatty acids are shown in the Table 6.6. The results were very different from our calculated profiles especially

ARA in the low ARA diet. It was supposed to be 0.20 g/100 g but the analysis result was 0.04 g/100 g. The ARA/EPA ratio was 0.12 g/100g and it was supposed to be 0.70.

**Table 6.6.** ARA and EPA analysis of control and low ARA diets

Fatty acid	Control diet	Low ARA diet
C20:4n-6	0.02	0.04
C20:5n-3	0.38	0.34
ARA/EPA	0.05	0.12

After some experimentation and discussion, it was found that this was due to several errors. ARA is not water soluble and it was originally added to water. An unknown amount may have precipitated out. We did also not correct for the impurities. The manufacturer had stated that the ARA was only 90% pure. The density of the ARA was 0.9 and no correction was made for this as ARA was added to the diet mix volumetrically. The animals were fed this incorrect diet for 45 days. After checking and corrected the errors, we obtained diets with the corrected ARA level in diets. The levels of ARA (J.K. Ju, Oceanic Institute) in the corrected diets are shown in the Table 6.7. The opihi were fed for another 50 days with these corrected ARA diets. Nine adult opihi were used for each diet. Feeding regime and assessing gonadal development were described as initial trial above. The latter experiment was conducted in coinciding with the natural maturation and spawning seasons Sep. 2013-Jan. 2014.

**Table 6.7.** Composition of formulated diet with various ARA levels.

Fatty acid	ARA/EPA ratio diet		
	Control	Low ARA	High ARA
ARA	0.02	0.24	0.39
EPA	0.34	0.34	0.56
ARA/EPA	0.06	0.70	0.70

Note: oil density 0.9 g/ml, and ARA impurity is 0.9 mg/ml

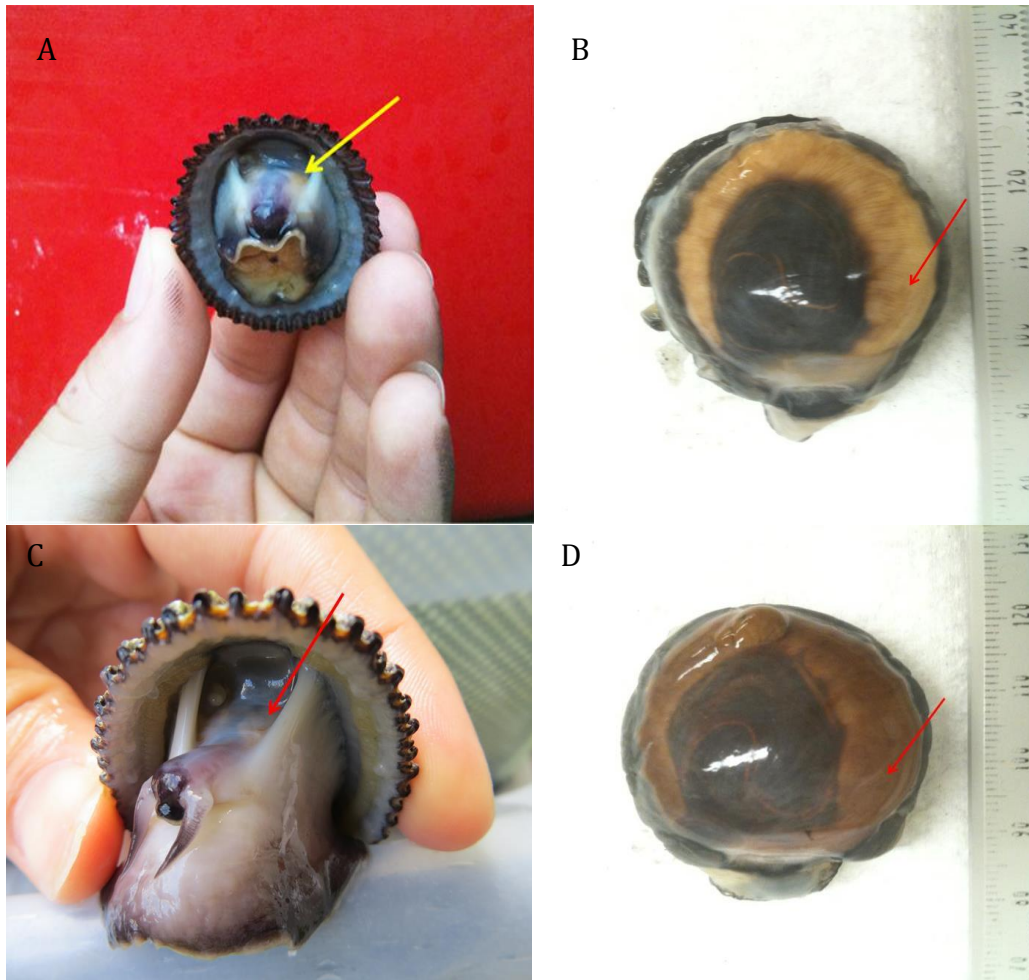
In the following trial, the final maturation of opihi was induced by using OvaRH (Syndel Laboratories Ltd. Canada) which is a synthetic salmon GnRH analog (sGnRHa). This synthetic hormone is an analogue of the native salmon GnRH consisting of pyroGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH<sub>2</sub> in which the amino acid position 6 (Gly) was replaced with Ala and the amino acid position 10 (Gly) was removed. Therefore, the amino acid sequences of the sGnRHa are pyroGlu-His-Trp-Ser-Tyr-Ala-Trp-Leu-ProNH<sub>2</sub> or Des-<sup>10</sup>Gly-<sup>6</sup>Ala-LHRH-ethylamine. It is believed that this synthetic salmon GnRH analogue produces the same biological function of LH and FSH secretion, but it prolongs stronger biological activity than the native salmon GnRH. The hormone was injected directly into the gonad of animal. Twelve opihi were tagged and weighed. The average total weight was 9.17±3.17 g. Each opihi received total of 5-7 injections, at 7 day intervals at dose of 250 ng/g body weight (BW). This particular dose was chosen because it was reported to be effective for maturation process in abalone (Nurrai *et al.*, 2010). The body weight was obtained from the equation of regression analysis of the relationships between the total weight with shell on and soft body tissue of sacrificed animals. The control was run without hormone injection. The animals were held on biofilm aquaria with water movement by an aquarium biofilter system (567 L per hour). Weekly, at least 2 animals were sacrificed and their gonads were immediately fixed in 10% formalin for histological examination and evaluation of the gonad somatic index. The experiment was conducted during the final maturation and spawning season (October 12<sup>th</sup> 2012- November 13<sup>th</sup> 2012)

Spawning induction trials. This study was conducted during the spawning seasons of opihi. Broodstock opihi (> 3 cm in shell length) were collected at the shoreline from a remote area on Oahu Island. They were held on biofilm aquaria (prepared as described in the previous chapter) for 1-2 days before being used. Broodstock that clung tightly to the side of the aquarium they were assumed to be healthy, and therefore used for the experiment.

Ripe broodstock definitions. Our earlier study showed that opihi could be sexed after killing and dissecting. Ripeness assessment could also be done after killing and dissecting. For live opihi, the gonads of ripe opihi could be seen when the animals were placed upside down on a table or put close to the edge of a substrate. When they try to attach to the substrate they move their foot towards the substrate and sometimes gonads may be seen. Males were identified if the animals had milky white gonad near the edge of shell, as shown by the arrow in the Figure 6.1 (panel A). The gonad of the ripe female was darker brown or darker green color, Figure 6.1

(panel C). The ovary may be seen with difficulty. We tried to assess the size of the gonad as an indicator of ripeness or GSI but this was hard to see clearly as seen in the Figure 6.1, panel B and D which are dissects. There was no selection for sex or ripeness for spawning studies and all animals collected from the wild were used. It was judged that sexing and ripeness determination was too difficult for live animals. In spawning studies, only about 50% of the broodstock spawned illustrating a 50% sexing or ripeness rate.

As indicated above, clear and quantitative determinations of ripeness and sex could only be made on the broodstock that died or were sacrificed. For prediction sake, we assumed that the GSI of the dead animals and their egg size (females) were similar to cohorts from a same-day collection that were kept alive. Our previous work showed that there was no statistically significant difference among the average GSI of animals during the spawning seasons ranging from  $22.9 \pm 12.4\%$  to  $31.8 \pm 7.72\%$ . Further statistical analysis for GSI of individual sexes showed that the average for ripe male and female were about  $30.3 \pm 2.99\%$  and  $24.2 \pm 12.3\%$  respectively. The egg size should be greater than  $110 \mu\text{m}$  in diameter and show a round shape. More than 80% of total full grown oocytes should be in the ovary. This was taken to define mature females. For the males, the sperm containing organs were milky white color and viscous. Active sperm were also confirmed under a compound microscopic.



**Figure 6.1.** Classification of sexually mature male and female opihi. A, live opihi before dissecting, the arrow shows a sign where mature male gonad could be identified. B, soft body tissue was removed off the shell, the mature male gonad (milky white) took up all the space around the digestive gland (dark color). This supports the location of mature gonad where it was seen as pointed in the picture A. C, live female opihi before dissecting, the arrow points where mature female's gonad would be seen. D, showing a dark brown mature female gonad with shell removal.

Two spawning methods consisting of hydrogen peroxide and salmon GnRH analog were employed.

Induction of spawning by using hydrogen peroxide. The first spawning trials with hydrogen peroxide followed the methods described by Morse *et al.* (1977) for abalone and

Corpuz (1983) for opihi. A relatively fresh purchased nonstabilized 30% H<sub>2</sub>O<sub>2</sub> (Acros, ACS reagent, Fisher Scientific) was used in our study. Although H<sub>2</sub>O<sub>2</sub> technique has been used to induce spawning in abalone but it is not entirely effective for all species. While there is still suspicious about the use of H<sub>2</sub>O<sub>2</sub> for spawning induction of opihi, we decided to test again with a lower concentration of H<sub>2</sub>O<sub>2</sub> than the level of Corpuz's report. Four different concentrations of H<sub>2</sub>O<sub>2</sub> were tested including 0.60 x 10<sup>-2</sup> %, 1.20 x 10<sup>-2</sup> %, 1.49 x 10<sup>-2</sup> %, and 1.80 x 10<sup>-2</sup> %. The control was run without H<sub>2</sub>O<sub>2</sub>.

Five to eight broodstock (approximate sex ratio, male:female = 1:1) were selected from the holding aquarium as described above and placed into a spawning jar containing 3 L fresh clean seawater for each trial. In other cases, only 5 to 7 opihi were used because the rough water conditions made it difficult to collect enough broodstock. The jar was gently aerated and pH in the spawning jar was first adjusted to pH 9-9.5 by 1M of Tris-base for about an hour. Thereafter, an amount of stock 6% of H<sub>2</sub>O<sub>2</sub> was slowly added to spawning jar to obtain the desired concentration. The stock 6% of H<sub>2</sub>O<sub>2</sub> was diluted right before use from concentrated 30% of H<sub>2</sub>O<sub>2</sub>.

The response of animals during and after exposure (in 24 hr) to each tested concentration of H<sub>2</sub>O<sub>2</sub> was observed and documented. The exposing time (5-45 min) of the broodstock to each tested concentration depended on the response of animals. When some abnormal behaviors such as mucus releasing and/or falling off the jar's wall were observed, the jar was immediately decanted. In all cases, the jar was rinsed with new fresh seawater a few times before spawning to safeguard the broodstock and to provide a non-toxic environment for larvae that are to come. Then the spawning activities were observed. The results will show that the highest number of spawners was induced at 0.6 x 10<sup>-2</sup> % and no mortality occurred in 24 hr after spawning. We used this level in further spawning trials. It was noticed that the response of animals after the first 24 hr was not observed for the first three trials at this concentrations and it was then recognized that animals could be affected up to a week in the last trial.

Induction of spawning by using sGnRH $\alpha$ . The second spawning experiment was conducted with sGnRH $\alpha$ . Two strategies of hormone injection were applied in this study. The first spawning treatment was an injection of one dose of 1,000 ng/g BW. This particular dose was reported to be effective on spawning induction on abalone (Nurrai *et al.*, 2010). Three trials (trial 1-3) were done during the spawning season, Jan 2013. The same dose injection was also

repeated twice (trial 4 and 5) in Dec 2013. An attempt was also made to induce final maturation and spawning by employing two injections when the GSI of broodstock was low (22-30%). The broodstock were injected with a priming dose of 250 ng/g BW. The second injection at dose of 1,000 ng/g BW was administered 24 hr after the priming injection (trial 6). Opihi used for this experiment were selected/not selected as described above. At least 5 animals were used for each trial. Each individual broodstock was weighed to obtain BW and received dose of hormone at 10:00 pm. The hormone was injected directly into the gonad of animal. After injection, the animals were placed into spawning jar (3 L/jar, 35 ‰). The jar was lined by plastic and with aeration supplied. The duration of spawning and number of spawned opihi were observed. The animals were injected with saline solution instead of hormone in the control.

**Handling of eggs.** After spawning the parents were returned to a maintenance aquarium. The air stone was removed away from the spawning container (jar). A few minutes later, the eggs sunk to the bottom of the container and they were first siphoned to another container through a net 205  $\mu\text{m}$  to remove all debris (released from the broodstock). The eggs were collected through a phytoplankton mesh (50  $\mu\text{m}$ ) and were rinsed few times with fresh clean seawater. At this point, we assumed that spawned eggs were already fertilized. They were then placed into a 1 L beaker with cleaned seawater and gentle aeration. Total spawned eggs were determined by randomly pipetting 3 mL from the beaker onto a watch glass or petri dish. The numbers of eggs were counted. This was repeated three times to get an average. The total number of spawned eggs was obtained by multiplying the average number by the total volumes in the beaker. Fertilized eggs were incubated in plastic container (3 L), which was gently aerated until they were hatched. The fertilization and hatching rates were then examined as follows.

**Fertilization and hatching determination.** Fertilization rate was assessed by estimating 100 eggs/beaker, and three 100 mL beakers were used. Approximately 2 hr after spawning, the numbers of eggs in the two-cell stage was counted. Eggs in the two cell stage were considered fertilized. The fertilization rate was calculated according to the formula: Fertilization (%) = (Number of two cells stage/Number of estimated eggs) x 100. Pictures were taken with a digital camera (Infinity analyze, version 4-6, 2008, Canada) attached to a compound microscope. Hatch rate was estimated in a similar way approximately 12 hr after spawning. Free swimming larvae were considered hatched. Hatching rate was calculated according to the following equation: Hatching (%) = (Number of free swimming larvae/Number of estimated eggs) x 100.

Handling of larvae. Larvae started hatching about 12-14 hr after spawning laboratory temperature (approximately 22°C). The air stone was removed from the incubated container for a few minutes and larvae swam up to the surface of the water because they are light sensitive. About the top 1/3 of seawater with hatched larvae in the container was siphoned or decanted into a new container through phytoplankton mesh (50 µm). The larvae were collected from the mesh. This process was repeated several times until all hatched larvae were collected. We believed that we had to siphon larvae to another container when they hatched. Otherwise, the larvae would die due to poor water quality caused by broken unfertilized eggs.

Algae preparation for larval settlement. An experiment aimed to successfully settle opihi larvae with different benthic diatoms and pelagic algae. Three benthic diatoms *Amphora montana*, *Nitzschia hantzschiana* and *Skeletonema costatum*, and two pelagic algae *Isocyrisis galbana* and *Pavlova lutherii* were used for larval settlement experiment. The algae were identified by suppliers. The two diatoms *Amphora* and *Nitzschia* were purchased from Kuehnle AgroSystems Inc. (2800 Woodlawn Dr., Ste. 281, Honolulu, HI, 96822). *Skeletonema* and pelagic algae were kindly provided from Dr. Audrey Asahi (Kawelo Marine Laboratory, University of Hawaii at Manoa). These benthic diatoms were reported to be the best diatoms for settlement of abalone larvae (Gordon *et al.*, 2006; Gapasin and Polohan 2005).

All materials and equipment were cleaned before use. The Table 6.8 shows each of materials and equipment and their appropriate cleaning technique.

**Table 6.8.** List of materials and equipments with an appropriate cleaning technique for algae culture.

No	Material	Applied cleaning method
1	Sea water	Autoclaved for 40 min at 125°C, then was filled through filter paper, 1.2µm
2	Erlenmeyer	Washed with soap and put in oven at 175°C for at least 30 min
3	Plastic bag for lining in plastic bottle	Clorox (3mL/L), or -80 degree Celsius
4	Cheesecloth	New and used cheesecloth needed to be covered by aluminum and put in oven, or can be put in flask and sterilized.
5	Pipettes and pipettes tips	Oven at 175°C for at least 30 min
6	Filters equipment, beakers, cylinder	Oven at 175°C for at least 30 min, before use
7	Air supply	Through syringes filter, 0.45 µm
8	Aeration tube, air stones, containers	Clorox (3mL/L)

The stock algae were cultured in 250 mL Erlenmeyer flask filled with F/2 medium (Guillard and Ryther, 1962). F/2 medium was prepared as shown in Table 6.9 and enriched with silica solution ( $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ , 30 mg/L) for *Skeletonema* diatom only. Another alternative commercial medium was also used, named Micro Algae Grow, which is a modified from Guillard F/2, and was purchased from Florida Aqua Farms Inc. (33418 Old Saint Joe Road, Dade City, Florida 33525 <http://florida-aqua-farms.com/shop/micro-algae-grow/>). A stock culture of diatoms (5mL) was added to 45 mL autoclaved seawater, containing F/2 medium in each flask. The diatoms *Amphora* and *Nitzschia* were at a density of  $3.0 \times 10^5$  cells/mL and  $1.5 \times 10^5$  cells/mL. They were incubated for 5-7 days in a shaded area with about 5,000 Lux of light. When the cell density reached approximately  $3.0 \times 10^5$  -  $4.5 \times 10^5$  cells/mL and  $1.5 \times 10^5$  -  $2.5 \times 10^5$  cells/mL for

*Amphora* and *Nitzschia* respectively, they were transferred to aquaria. *Skeletonema*, *Pavlova*, and *Isochrysis* were cultured and used for growout aquaria.

**Table 6.9.** F2 medium preparation for cultivation of algae.

Nutrients	Concentration (mg/L seawater)
NaNO <sub>3</sub>	75.0
NaH <sub>2</sub> PO <sub>4</sub> . H <sub>2</sub> O	5.00
Silicate solution, Na <sub>2</sub> SiO <sub>3</sub> .9H <sub>2</sub> O	30.0
Microelement stock solution	
Na <sub>2</sub> C <sub>10</sub> H <sub>14</sub> O <sub>8</sub> N <sub>2</sub> .H <sub>2</sub> O (Na <sub>2</sub> EDTA)	4.16
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.01
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.01
FeCl <sub>3</sub> .6H <sub>2</sub> O	3.15
MnCl <sub>2</sub> .4H <sub>2</sub> O	0.18
Na <sub>2</sub> MoO <sub>4</sub> .H <sub>2</sub> O	0.006
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.022
Vitamin solution	
Thiamin HCL (Vitamin B1)	100
Biotin (Vitamin H)	0.50
Cyanobalamin (Vitamin B12)	0.50

Larval settlement aquaria preparation. Six glass aquaria (50cm x 25cm x 30 cm; length x width x height) were thoroughly cleaned with soap and rinsed with commercial bleach solution, 3 mL/L Clorox (6.0 % sodium hypochloride). They were then dried in air for an hour. Each of aquaria was filled with 26 L fresh seawater filtered through phytoplankton net (25 µm). A rack consisting of two settlement plates (40 cm x 20cm; length x width) was made from clear polycarbonate corrugate roof sheet and was put into the each aquarium. This settlement substrate type was selected because it was a preferred plate for abalone larvae (Cecilia Viljoen, Big Island Abalone Company). It was also reported as one of the most suitable substrate for

metamorphosis of abalone larvae (Gapasin and Polohan 2005). Two small pieces of plate-substrate (4cm x 6 cm; length x width) were also placed into each aquarium at the same time with the rack. This will be used for experimental observation of larval settlement in the beaker. Each aquarium was covered with a clear plastic sheet secured with a string. The seawater was chlorinated with 78 mL Clorox (3mL sodium hypochlorite per L sea water), then aerated vigorously for 24 hr. The air was filtered through a syringe filter (0.45  $\mu$ m, Fisher Science). Seawater in each aquarium was neutralized by sodium thiosulfate (0.15 g/L) and aerated again for another 24 hr.

Six aquaria were inoculated with a combination of diatom and pelagic algal species. Our preliminary observations showed that all opihi larvae were dead in the second day in a clear water column in beakers after hatching and no survivors were obtained after 4 days in beakers containing only benthic diatoms. This suggested the necessity of pelagic algae in the rearing tank. In addition, literature study on larvae settlement of abalone showed that a combination of benthic diatoms seemed to induce a significantly higher larvae settlement and metamorphosis than a single benthic diatom (Searcy-Bernal *et al.*, 1992; Gapasin and Polohan 2005; Gordon *et al.*, 2006). The first aquarium was *Amphora* (initial cell density of 1,300 cells/mL) and *Palova* (initial cell density of  $18 \times 10^3$  cells/mL), the second aquarium contained *Nitzschia* (2,600 cells/mL) and *Palova* at the same density, the third one was *Skeletonema* (3,000 cells/mL) and *Palova* at the same density, the fourth one with *Amphora*, *Nitzschia* (1:1 proportion at about 2,600 cells/mL for each species) and *Isochrysis* ( $18 \times 10^3$  cells/mL), the fifth one was *Nitzschia*, *Skeletonema* (1:1 proportion at about 2,600 cells/mL) and *Isochrysis* ( $18 \times 10^3$  cells/mL) and the sixth one was *Amphora*, *Skeletonema* (1:1 proportion at about 3,800 cells/mL) and *Isochrysis* ( $18 \times 10^3$  cells/mL). Diatom cultures were allowed to grow on the plate-substrates for at least 4-7 days until microalgal film that was observed under a shaded area where there was about 10,000 Lux of sunlight (Fig. 6.2). The temperature was ranging from 23-28°C.



**Figure 6.2.** Experimental diatoms culture aquaria under shade area, where it has about 10,000 Lux.

The cell density on plate-substrate and pelagic algae in the water column were estimated before placing the opihi larvae. To estimate the diatom density grown on plate-substrate, about  $1.0 \text{ cm}^2$  of diatoms grown on each plate-substrate was sampled and scraped off with a soft plastic ruler, and then pipetted into a centrifuge tube and fixed with 5% formalin phosphate. The cell density was counted by using hemacytometer (Hausser Scientific, 935 Horsham Rd. Suite C, Horsham, PA 19044) under a compound microscope. The pelagic diatoms were also counted in a similar way.

Experimental larvae. The larvae used for this experiment were obtained from the GnRH spawning trial. About 6 hr after hatching, about 800 larvae were placed into each of the aquaria above, which had thin film diatoms grown on the plate-substrate for 7 days and were slightly cloudy with pelagic algae. Water temperature was monitored every day and dissolved oxygen was measured every two days for the first 10 days. In the meanwhile, the experiment was also conducted in a 250 mL beaker in laboratory. Seawater (150 mL) was obtained from each of the diatom aquaria above with a piece of plate-substrate (4cm x 6 cm; length x width) with diatom growth. The piece of plate-substrate was prepared at the same time as others. Two beakers were used for each type of diatom diet. Thirty larvae were put in each beaker. The beakers were covered with parafilm and were gently aerated via Pasteur pipette connected syringe filter. A 24 hr fluorescent light (3,000 Lux) was provided for the first two days to maintain diatom grown,

and then turned off at night time from the third day. The control was run in fresh clean seawater. Larvae could be seen in the water column as objects that appeared to be swimming up to Day 2. At Day 3 settled larvae were seen as spat on clumps of diatoms. The numbers of settled larvae were counted and presented as percentage of settlement larvae. Their identification was confirmed by microscope.

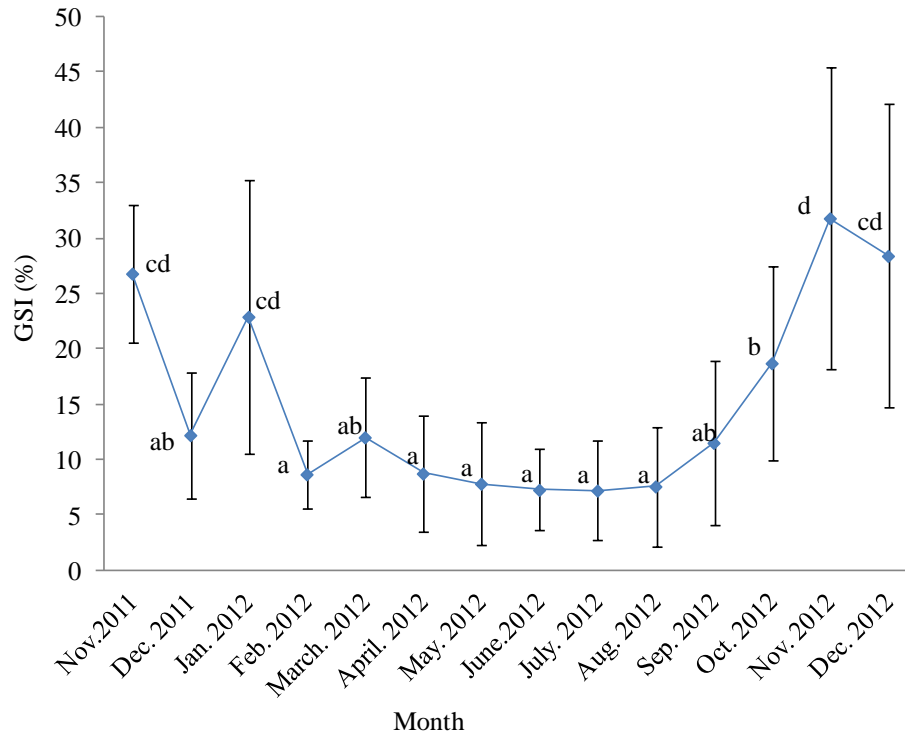
## **RESULTS**

Spawning season. Table 6.10 shows the average size and GSI of animals sampled monthly. Mean shell length ranged from  $2.86 \pm 0.48$  cm to  $4.26 \pm 0.59$  cm. Total weight and soft body tissue (body weight) varied seasonally; a ratio of body weight to total weight was approximate 38.7%. The average GSI ranged from  $7.19 \pm 4.51\%$  to  $31.8 \pm 7.72\%$  and statistics will be shown later.

**Table 6.10.** Average size and GSI of sampled opihi for the reproductive cycle study from November 2011 to December 2012, data values from n individuals are presented as Mean±SD.

Date of sampling	n	Shell length (cm)	Total weight (g)	Body Weight (g)	Gonad weight (g)	GSI (%)
11/12/11	13	4.26±0.59	15.3±5.49	5.93±2.43	1.68±0.92	26.8±6.27
12/04/11	30	3.46±0.51	7.67±3.63	2.54±1.39	0.31±0.22	11.7±5.22
01/31/12	17	3.55±0.96	9.31±5.74	2.97±2.40	0.93±1.06	22.9±12.4
02/28/12	27	3.11±0.31	4.83±1.74	1.86±0.66	0.17±0.09	9.05±3.65
03/28/12	16	2.86±0.48	4.68±2.62	1.64±0.71	0.20±0.09	12.0±5.41
04/24/12	12	3.26±0.58	5.20±2.47	1.81±1.03	0.15±0.14	8.73±5.31
05/28/12	12	3.27±0.56	5.20±2.47	2.90±3.50	0.14±0.13	8.10±5.25
06/28/12	17	3.17±0.16	5.88±0.91	2.19±0.55	0.17±0.11	7.25±3.69
07/21/12	23	3.25±0.43	5.98±1.67	2.21±0.78	0.16±0.11	7.19±4.51
08/03/12	12	3.95±0.50	9.49±3.91	3.47±1.50	0.30±0.28	7.56±5.37
09/11/12	20	3.43±0.44	5.43±2.19	1.89±0.91	0.28±0.29	11.5±7.43
10/05/12	21	3.71±0.92	7.07±5.38	2.86±2.01	0.65±0.73	18.7±8.77
11/25/12	27	3.96±0.49	8.75±3.21	3.73±1.47	1.31±0.73	31.8±7.72
12/30/12	19	3.96±0.83	10.2±8.64	4.10±3.77	1.52±1.94	28.4±3.75

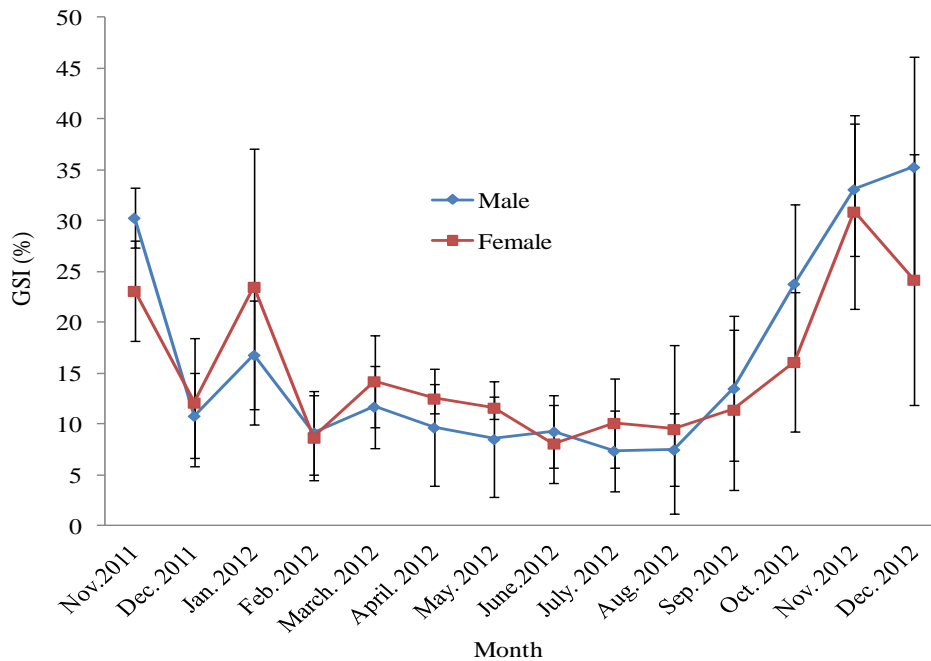
Figure 6.3 is a graphical representation of the data shown above. It shows GSI values monthly from November 2011 to December 2012. One-way ANOVA analysis, followed by Tukey test revealed that there was a significant difference ( $P < 0.001$ ) in monthly GSI of animals during the study period. The highest average GSI of *C. sandwicensis* was noticed between November and January. This suggests the spawning seasons of opihi would occur from November to January. The GSI in Dec. 2011 was outlier. GSI dropped in February, suggesting the end of the spawning period. The lowest GSI was observed from March to August, but GSI gradually increased from September to November.



**Figure 6.3.** Seasonal changes in GSI of opihi *C. sandwicensis*.

<sup>a,b,c,d</sup> different letters indicate significant difference in the average percentage GSI of animals (n=12-30) sampled from November 2011 to December 2012.

The pattern of gonad development of males and females *C. sandwicensis* is shown in the Figure 6.4. Overall, a similar trend was found in GSI of both sexes throughout the study. Statistical analysis showed that there was no significant ( $P > 0.05$ ) difference between GSI of males and females caught in the same month. The data seem to be consistent with synchronized spawning of male and female in the wild.



**Figure 6.4.** Seasonal changes in GSI of males (n = 7-19) and females (n = 8-14) opihi sampled from November 2011 to December 2012.

The GSI of the individual sexes during study period (Table 6.11) was further carefully examined. Statistical analysis showed that there was a significant ( $P < 0.0001$ ) difference in GSI of males among the monthly measurements from November 2011 to December 2012. The highest GSI of male was 35.3% for December 2012, and this did not differ significantly compared to November 2012 (33.1%) and November 2011 (30.3%), but differed significantly compare to other monthly measurements. This suggests that the minimum average mature GSI for male should be about 30.3%. The highest average GSI of female was 30.9% in November 2012, but there was no significant difference with that GSI of animals in Dec 2012 (24.2%) and January 2012 (26.5%). This suggests that the minimum average mature GSI for female should be about 24.2%. The GSI of males and females in December 2011 was an outlier or this may be due to spawning in this limited group.

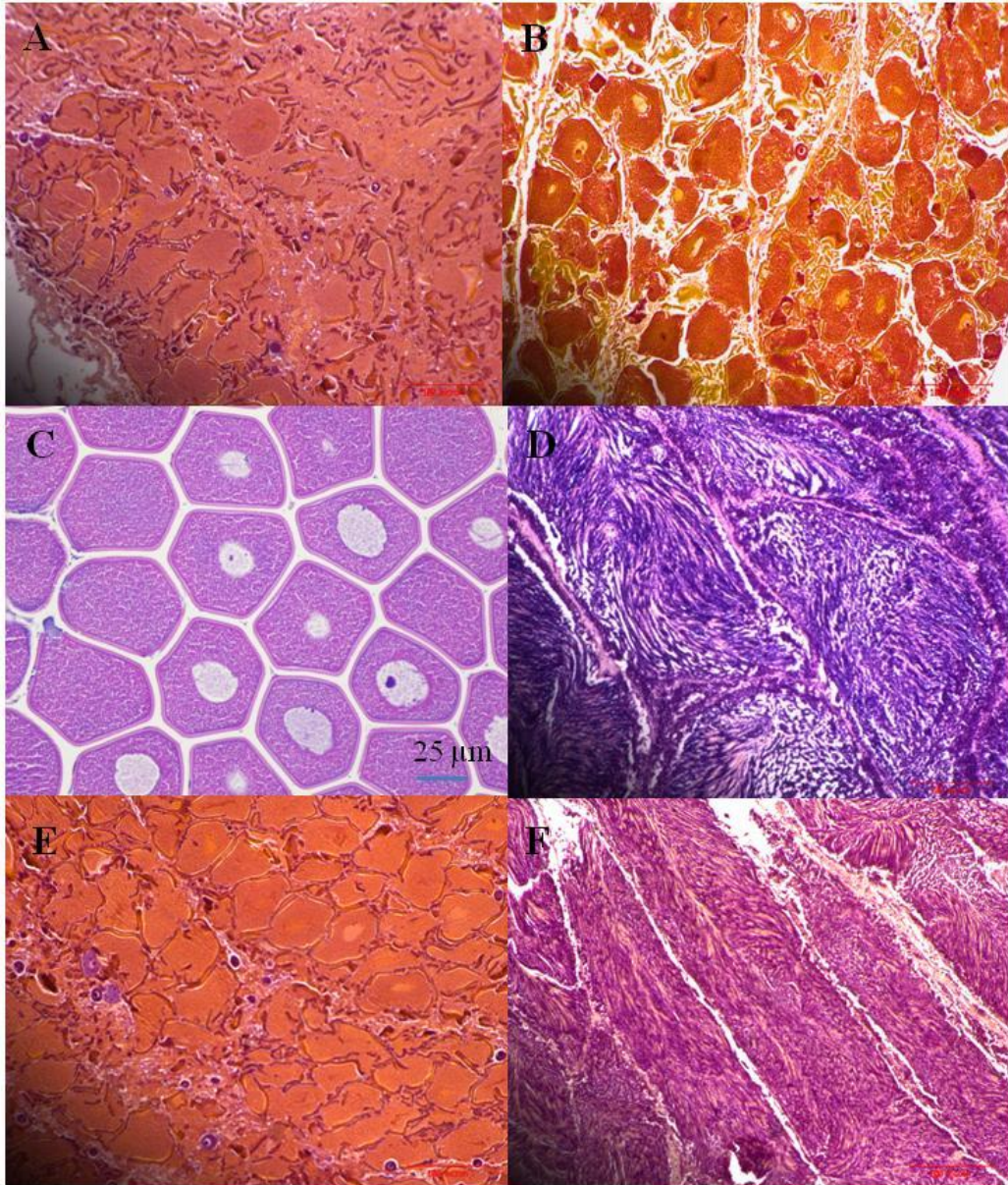
**Table 6.11.** GSI of male and female opihi throughout the study.

Month	GSI (%)	
	Male	Female
Nov. 2011	30.3±2.99 <sup>cd</sup>	23.1±4.95 <sup>abc</sup>
Dec. 2011	10.8±4.20 <sup>a</sup>	11.0±4.67 <sup>a</sup>
Jan. 2012	16.8±5.31 <sup>ab</sup>	26.4±11.0 <sup>bc</sup>
Feb. 2012	9.16±4.09 <sup>a</sup>	8.65±4.14 <sup>a</sup>
March 2012	11.7±4.04 <sup>a</sup>	14.4±4.60 <sup>ab</sup>
April 2012	9.66±5.79 <sup>a</sup>	11.5±0.87 <sup>ab</sup>
May 2012	8.51±5.64 <sup>a</sup>	11.6±1.06 <sup>ab</sup>
June 2012	9.26±3.5 <sup>a</sup>	10.1±4.28 <sup>a</sup>
July 2012	7.36±4.00 <sup>a</sup>	10.1±4.36 <sup>a</sup>
Aug. 2012	7.47±3.62 <sup>a</sup>	9.49±8.32 <sup>a</sup>
Sep. 2012	13.5±7.08 <sup>ab</sup>	11.4±7.88 <sup>ab</sup>
Oct. 2012	23.8±7.78 <sup>bc</sup>	16.1±6.86 <sup>abc</sup>
Nov. 2012	33.1±6.52 <sup>cd</sup>	30.9±9.52 <sup>c</sup>
Dec. 2012	35.3±10.8 <sup>d</sup>	24.2±12.3 <sup>abc</sup>

<sup>a,b</sup> the same letters indicate no significant difference in the average percentage GSI of male and female in the same column

Figure 6.5 is a set of histology slides of gonad sections of opihi through the final maturation, spawning, and resorption phases. The Figure 6.5A showed that unclear oocytes formation and eggs being undistinguishable from ovary-cell wall in the ovary of female that was collected in May 2012, before the final maturation season. These characteristics are considered as resting stages which were frequently observed from April to August. In the following Figure 6.5B, multiple development stages were seen in the same ovary of female was collected in October (during the final maturation season and before GSI peak). Most of the oocytes were in the early stages and late development stages. This indicates that the animals were immature during this period. Maximum ripeness for both sexes was observed in November (11/25/12) and the cross-section of ovary and testis are shown in the Figure 6.5C,D. About 80% of oocytes

were ripe in ovary with GSI of 24.8%. Similar observation was made for gonad of the male (Fig. 6.5 D). The testes were densely packed with spermatozoa which appeared dark blue stained by hematoxylin. In the resorption phase, multiple development stages were seen in the same ovary of female was collected in February (02/28/12), the oocytes seemed deformed (Fig. 6.5E). Sperm were less densely in the male gonad (Fig. 6.5F).



**Figure 6.5.** Cross-sections showing stages of ophiu gonad development.

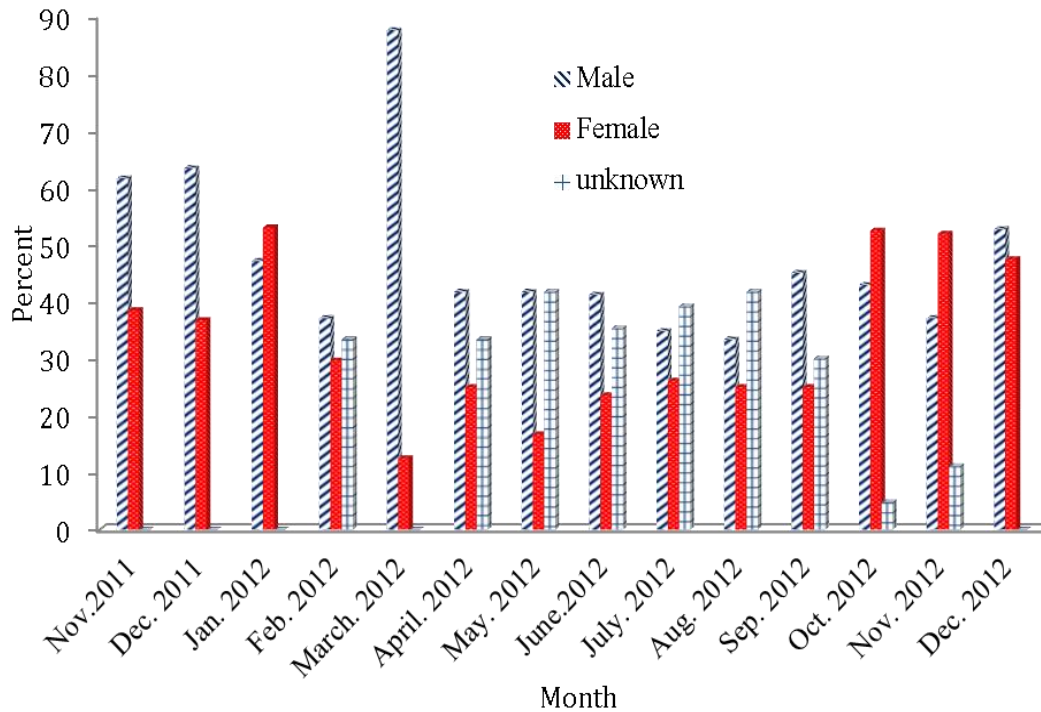
A, resting development stages of female (May 2012). B, most oocytes in early development stages in ovary of female (October 2012). C, ripe stage and some oocytes were still in late development stage (November 2012). D, mature male gonad with dark blue stained by hematoxylin (November 2012). E, oocytes deforming shape in the ovary (February 2012) and F, spermatic in gonad of male (February 2012).

The total number of eggs in several mature opihi was estimated (Table 6.12). Fecundity (F) or total eggs counted of mature female opihi ranged 42.1 to 157 million eggs per kg body weight (BW). Fecundity was linearly correlated to body weight ( $P = 0.019$ ). The respective relationship is best described by the equations:  $F = 28.4BW - 77.3$  ( $R^2 = 0.96$ ). The number of eggs was generally proportioned to body weight rather than shell length.

**Table 6.12.** Representative potential fecundity of several mature opihi.

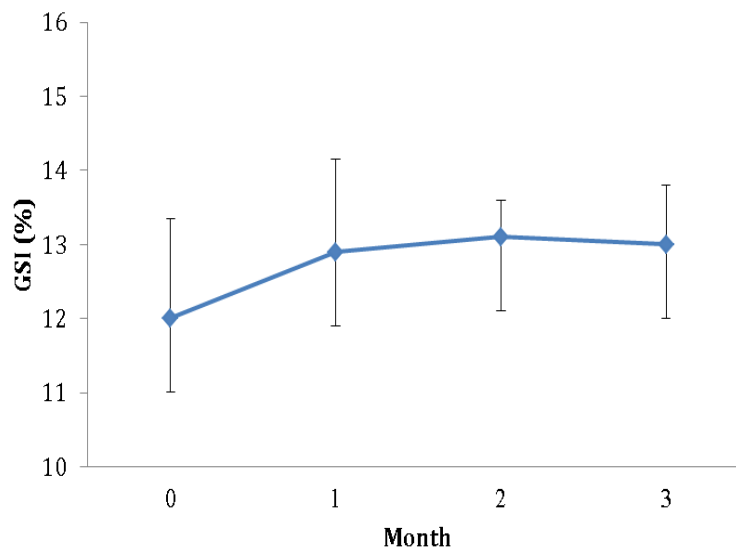
Animal	Shell length (cm)	BW (g)	GW (g)	GSI (%)	Fecundity (total eggs/BW)	Fecundity (No. eggs/g BW)
1	4.50	5.47	1.47	26.9	412,986	75,500
2	4.40	4.33	1.01	23.3	182,034	42,080
3	4.60	6.69	2.41	36.0	737,419	110,227
4	4.34	5.33	1.87	35.0	835,632	156,779
5	4.57	6.83	1.78	26.1	310,879	45,515

Figure 6.6 shows the distribution of sexes of opihi collected monthly during reproduction cycle study. Among a total of 266 wild caught opihi, there were 126 males, 92 females and 48 of whose sex were unknown. The sex ratio was approximately 2:1 for male:female. However, the ratio appeared to be 1:1 during spawning season (November to January) and all of the animals sexed after dissection. As indicated previously sexing was not done on live animals and ripeness as measured by GSI is almost impossible during spawning studies. Failures in sexing the animals occurred from April to September due to immature gonad, which was also associated with the lowest GSI (lower than 3%).



**Figure 6.6.** Monthly distribution of sexes of *C. sandwicensis* from November 2011 to December 2012.

Induction of final maturation. Attempts were made to mature opihi in the laboratory. Our first results showed that the animals did not reach the final maturation when they were fed with the growout diet. Figure 6.7 showed that GSI remained constantly for a three month period. The GSI was ranging from 12% to 13%, while the GSI greater than 24% would be characterized for ripe broodstock in earlier study. The analysis of fatty acid profile of the growout diet (Table 6.4) showed that ARA level was 0.02 g/100g and 0.52 g/100g for EPA, and relative ARA/EPA was only 0.04. This predicted that ARA level in the diet might be insufficient for the final maturation. On the other hand, it is noted that this experiment was not conducted in the natural final maturation season. It was conducted between May 2012 and Aug. 2012 under natural daylight and temperature conditions. This indicates that natural photoperiod and temperature at that time may not be good favorable conditions for the maturation process of opihi.



**Figure 6.7.** Monthly GSI change of broodstock (n=3) fed with growout diet

A follow up maturation trial with a manipulated ARA/EPA ratio of 0.70 in diet was conducted with low (0.20 g/100 g) and high ARA (0.33 g/100 g) diets. The analysis of the fatty acid profile of the low ARA diet showed that the ARA level was incorrect. It was 0.04 g/100 g and very much lower than needed level of 0.20 g/100 g. The relative ARA/EPA ratio in this diet was 0.12, which was much lower than the desirable 0.70. This was a mistake due to not correct the impurity of ARA and the animals were fed with these incorrect diets of ARA for 45 days (Sep. 23<sup>th</sup> 2013-Nov. 05<sup>th</sup> 2013) and the result of GSI are shown in Table 6.13 and Figure 6.8. GSI of animals that were fed with low ARA and high ARA diets were doubled compared to the control over the 45 days period. No eggs size were measured for the control because no female was sampled among the controls. In these trials animals that died spontaneously were used as animals needed to be sacrificed for GSI determination and animal use was very conservative. There was no significant difference ( $P > 0.05$ ) between egg size of animals that were fed with low and high ARA diets.

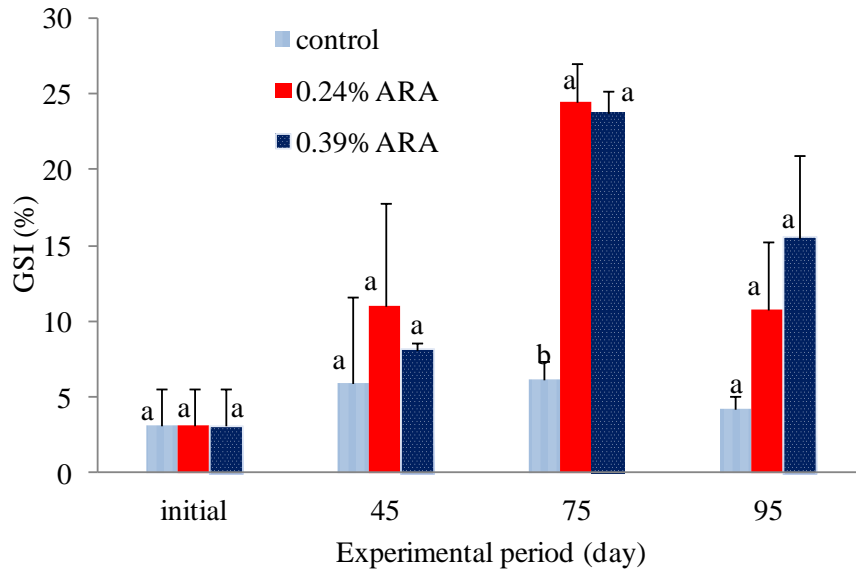
In a second phase the animals were fed with correctly made diets of 0.24 g/100 g ARA diet (Low ARA diet) and 0.39 g/100 g ARA for the high diet with the same ARA/EPA ratio of 0.70. Both diets produced a greater gonad development up to day 75 (November 6<sup>th</sup> 2013-December 05<sup>th</sup> 2013). The GSI of the animals fed with both correct low and high ARA diets increased

three times more than the GSI of animals that were fed with the control diet and about two times more than those GSI of animals that were fed with incorrect low and high ARA diets in the first 45 days. There was no significant ( $P > 0.05$ ) difference in the size of egg between the correct low ARA and high ARA diets. No female was sampled at this measurement for control. The GSI of animals decreased in all diets after 95 days (Dec. 6<sup>th</sup> 2013-Jan. 06<sup>th</sup> 2014). At this point in time the animals could have undergone reabsorption. It is noticed that this experiment was conducted in coinciding with the natural maturation and spawning season of opihi in the wild. No female was sampled for low ARA diet at the 95 days measurement. There was one female for the high ARA diet but the eggs were deformed and was difficult to measure eggs size exactly under compound microscope. A female was sampled for the control diet but the gonad was immature.

**Table 6.13.** Gonadal somatic index and egg size of animals fed various ARA levels over 95 days.

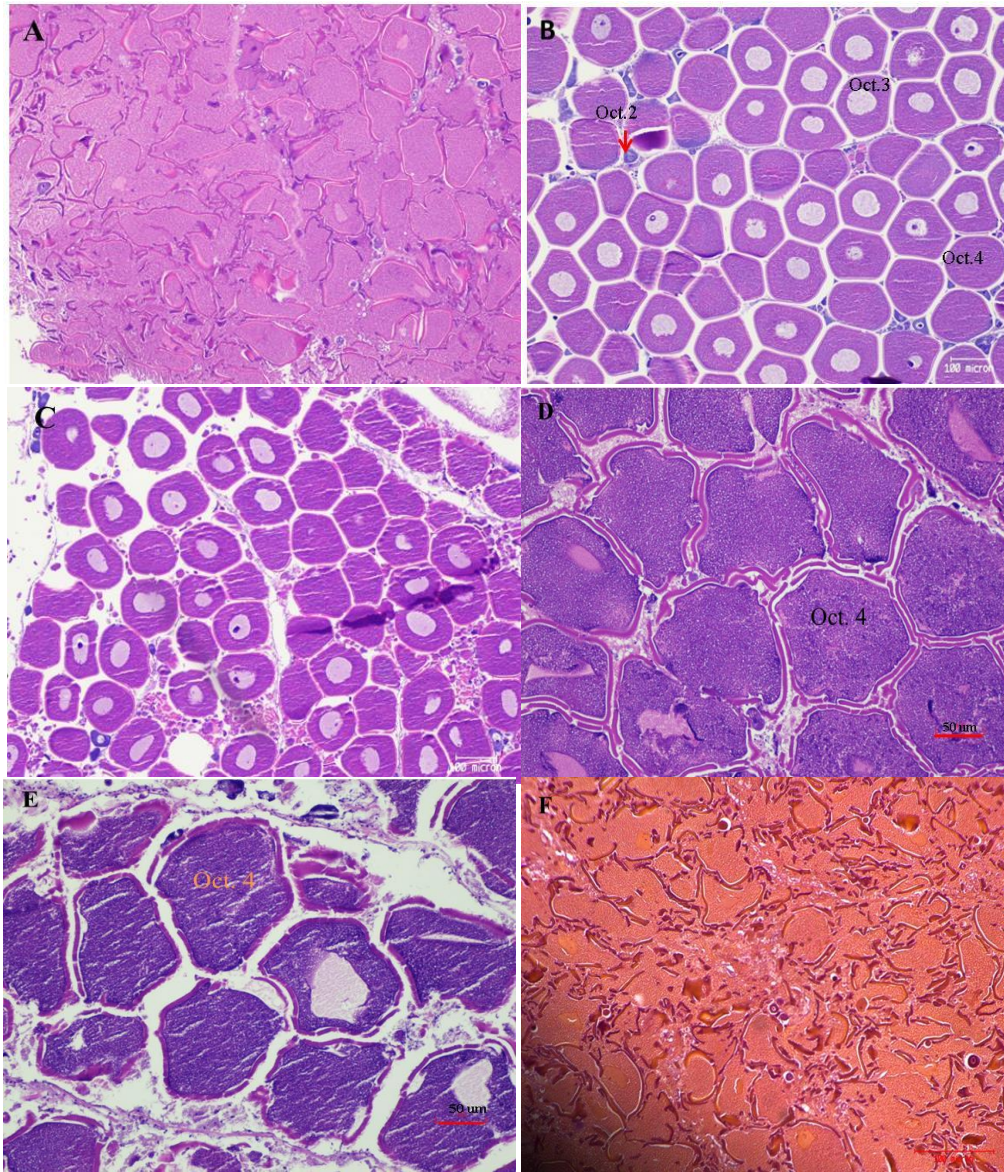
Day	Measurement	Control	Low ARA	High ARA
Initial	GSI (%)	3.10±2.48	3.10±2.48	3.10±2.48
45	GSI (%)	5.94±5.65	11.0±6.82	8.13±0.52
	Egg size (µm)	-	118±9.71 <sup>a</sup>	121±9.42 <sup>a</sup>
75	GSI (%)	6.11±1.25	24.5±2.52	23.7±1.43
	Egg size (µm)	-	123±4.23 <sup>a</sup>	121±5.93 <sup>a</sup>
95	GSI(%)	4.21±0.82	10.8±4.47	15.5±5.47

<sup>a,b</sup>the same letters in row indicate no significant difference in eggs sizes, the empty grids indicate no egg was observed.



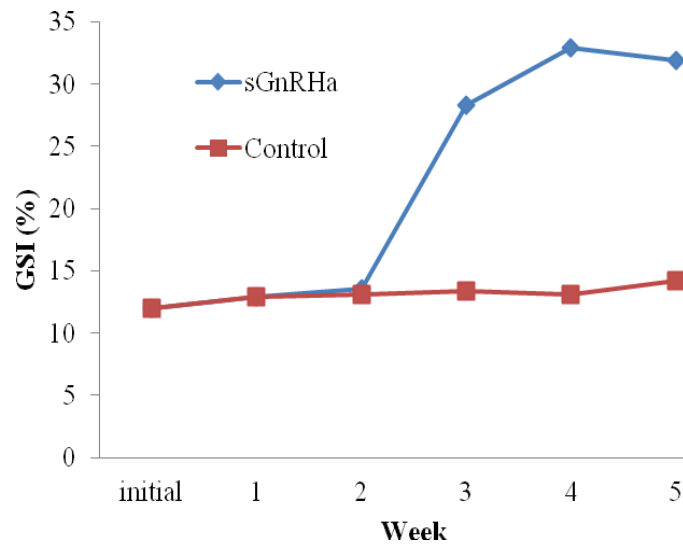
**Figure 6.8.** Gonadal somatic index of opihī *C. sandwicensis* fed various ARA diets. Low ARA (0.24% ARA) diet and high ARA diet (0.39% ARA). <sup>a,b</sup> different letters indicate significantly difference in mean GSI (n =2-3) of opihī within the same date of sampling.

A histological study confirmed the gonad development of animals when they were fed with ARA diets (Fig. 6.9). The initial female's gonad contained slack oocytes, which did not have defined shapes (panel A, Fig. 6.9). The cross-section of ovaries of animals that were fed with the incorrect low and high ARA diets showed that the oocytes were clearly formed but contains multiple stages and various sizes in the first 45 days (Fig. 6.9 B and Fig. 6.9 C). These indicate that the gonads of animals have not yet developed to the maturity stage at this time. Stage 2 is an early maturity stage with small oocyte diameter (7-10  $\mu\text{m}$ ). In stage 3, oocyte cells have a large central lumen and were most frequently seen (about 48-55%). A few oocytes (6-10%) have already turned into maturity stages. After being fed with the correct diet, the ovary cells grew in both diets up to 75 days period. Fig. 6.9D and Fig. 6.9E showed that most of the eggs were in the stage 4 (about 73%), which was defined as a ripe stage, and the egg's membrane were separated and round shape. Eggs were deformed the shape after 95 days (Fig. 6.9F).



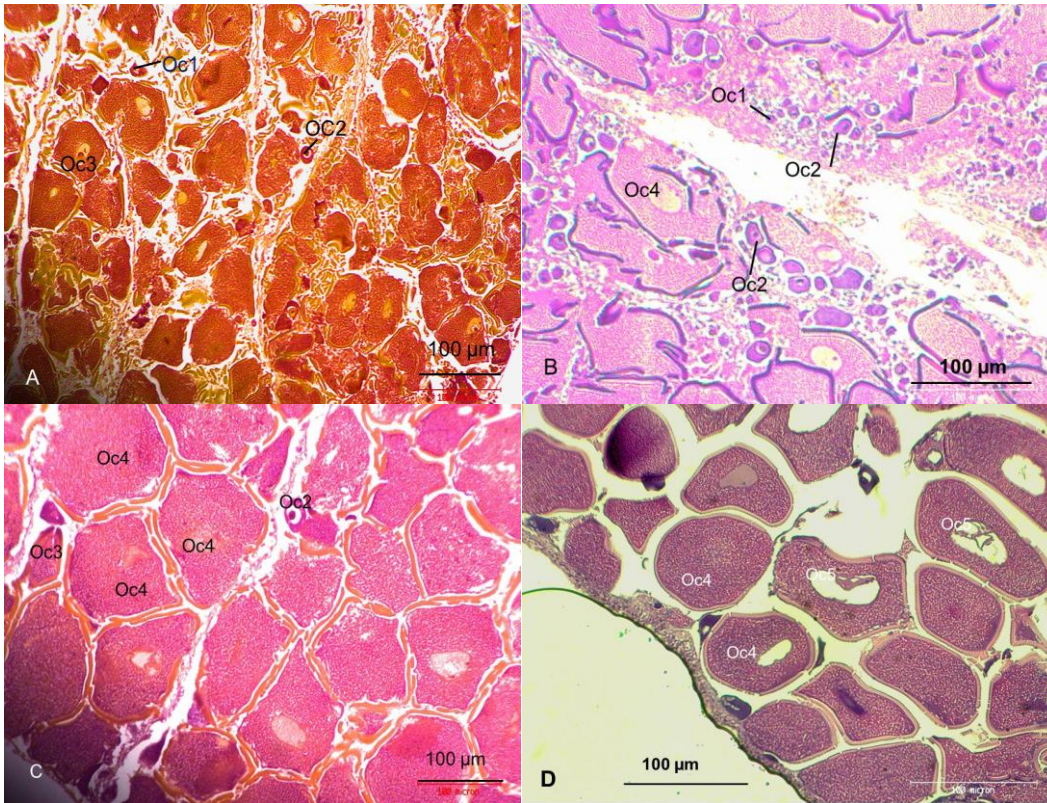
**Figure 6.9.** Cross-section ovary of opihi *C. sandwicensis* stained with hematoxylin-eosin. A, initial immature female's gonad. B, the gonad of female that fed with incorrect low ARA diet for 45days, stage II oocyste (Oc2). C, the gonad of the female that fed high incorrect ARA diet for 45 days. D, ovary at 75 days fed with correct low ARA diet (40 x). E, ovary at 75 days fed with correct high ARA diet, stage IV Oocytes (Oc4) and F, female gonad at 95 days fed with high ARA diet.

Hormonal induction of final maturation. Hormonal injection with salmon GnRH<sub>a</sub> successfully induced the final maturation in opihi. The results showed that the gonads of animals were rapidly developed after three weeks injection (Fig. 6.10). GSI of opihi increased rapidly from initial 12.0% to 28.3% after the third injection and reached 32.9% for the final maturation stage after the fourth injection. The spawning induction did not try due to the lack of animals. These data were higher than the control group without hormones injection. The GSI of animals in the control group remained constantly through the experiment. The experiment was conducted during the maturation and spawning seasons (October 12<sup>th</sup> 2012- November 13<sup>th</sup> 2012). This indicates that the natural environmental factors such as photoperiod and/or temperature may also facilitate the final maturation.



**Figure 6.10.** Gonadosomatic growth of opihi by hormone induction

Sections of gonads showed proliferative stages in maturation when treated with hormone (Fig. 6.11). The control group animals had various ovary sizes (Fig. 6.11B), whereas after 4 week of hormones treatment the gonad developed rapidly and most of oocytes were in the ripe stage (Fig. 6.11C,D).



**Figure 6.11.** The effect of hormones on gonad maturation (hematoxylin-eosin staining). A, initial week without hormone injection showing a proliferative stage with several stage I (Oc1), II oocysts (Oc2). B, Control group without hormone injection after 5 weeks. C, hormones treatment after 4 week and D, gonads maturation stage after 5 week injections with  $250\text{ng}\cdot\text{g}^{-1}\text{BW}$ , showing a fully mature stage IV Oocytes (Oc4).

Spawning induction by using  $\text{H}_2\text{O}_2$ . Spawning induction by using hydrogen peroxide is traditional for abalone and was tried with opihi (Corpuz 1983), limpet *Lottia digitalis* (Kay and Emler 2002). We eventually found that there was a non-specific toxic effect on the animals. The first range finding spawning experiments was to test different  $\text{H}_2\text{O}_2$  concentrations to determine the effective  $\text{H}_2\text{O}_2$  level for spawning induction. The results are shown in the Table 6.14. There was 43 % spawning of opihi for both concentrations  $0.6 \times 10^{-2}$  and  $1.2 \times 10^{-2}$  % of  $\text{H}_2\text{O}_2$ . There were no visual effects for the first 35 minutes, no mortality occurred within 24 hr after spawning at the concentration of  $0.6 \times 10^{-2}$  %, whereas 37% mortality rate was monitored in 24h after exposure to  $1.2 \times 10^{-2}$  %  $\text{H}_2\text{O}_2$ . High mortality for both trials of  $1.49 \times 10^{-2}$  % and  $1.80 \times 10^{-2}$  %

H<sub>2</sub>O<sub>2</sub> were observed at 71% and 75% respectively. The animals commenced releasing mucus and falling off the side of wall of the jar about 5 min after being exposed to these two concentrations of H<sub>2</sub>O<sub>2</sub>. They died in 24 hr after being treated with peroxide. There was no spawning at 1.49 x 10<sup>-2</sup>%, but there was 13% spawning for the 1.80 x 10<sup>-2</sup>% trial. Thus, this experiment showed that the lowest level of 0.6x10<sup>-2</sup>% H<sub>2</sub>O<sub>2</sub> seemed to be effective and safe, but further experiments were needed with more replications to confirm the use of H<sub>2</sub>O<sub>2</sub> and this was followed up on below.

**Table 6.14.** The effect of various H<sub>2</sub>O<sub>2</sub> concentrations on spawning of opihi.

H <sub>2</sub> O <sub>2</sub> concentration (%)	n	Time of expose (min)	Mortality rate (%) after 24 hr	Percent of spawn
Control (10/30/12)	5	-	-	-
0.60 x 10 <sup>-2</sup> (10/30/12)	7	35	None	43
1.20 x 10 <sup>-2</sup> (11/16/12)	7	15	37	43
*1.49 x 10 <sup>-2</sup> (10/30/12)	5	5.0	71	0.0
1.80 x 10 <sup>-2</sup> (11/16/12)	8	5.0	75	13

\*based on report of Corpuz (1983)

As indicated, spawning induction of opihi should be successful at 0.60 x 10<sup>-2</sup> % H<sub>2</sub>O<sub>2</sub> (Table 6.15). It was shown that this concentration of peroxide did not lead to mortality after 24 hr exposure. The toxic effect of H<sub>2</sub>O<sub>2</sub> after the first 24 hr was not paid attention to for the first three trials (trial 1-3). This was a failure in research notebook record keeping and fuzzy recollection has it that animals died periodically after the 24 hr. However, it was noted that all animals died in a week after exposure in the last trial. No spawning was observed for the control trial. Spawning rates varied ranging from 14% to 80% among the other trials. This was a problem. Fecundity also varied ranging from 26,987 eggs (9,242 eggs per g BW female) to

769,956 eggs (86,081 egg per g BW female). The average hatching rate was 16%. Maturity of eggs was also variable. Although about 40% spawning was obtained in Trial 4, most of these eggs seemed to be immature, the average spawned eggs size being  $100 \pm 6.69 \mu\text{m}$ . Thus, the spawning induction by  $\text{H}_2\text{O}_2$  at  $0.60 \times 10^{-2} \%$  seemed to be effective but cannot be recommended for future use due to loss of broodstock using this method. They released gametes in early in the morning from 3:00-5:00AM, most the cases the spawners were visually observed when they spawned.

**Table 6.15.** Spawning trial using  $\text{H}_2\text{O}_2$  at  $0.60 \times 10^{-2} \%$ .

Trial	n	No. of spawn	% spawn	Total eggs produced	Fertilization rate (%)	Hatching rate (%)
Control (11/20/12)	5	None	-	-	-	-
1 (11/20/12)	7	3 (1F, 2M)	43	26,987	23	7.0
2 (11/26/12)	7	1 (M)	14	-	-	-
3 (12/03/12)	5	4 (2F, 2M)	80	769,956	$36 \pm 6.6$	$35 \pm 12$
4 (11/19/13)	5	2 (1F, 1M)	40	50,000	Not determined	6.0

Notes; F: female; M: male

Spawning induction by using sGnRH $\alpha$ . Spawning induction of opihi by using the sGnRH $\alpha$  was better than hydrogen peroxide method, yielded larvae for further larval rearing trial, and was considered a method worth pursuing (Table 6.16). Broodstock were not killed during this treatment and some insight was gained on broodstock quality. Some larvae survived to Day 9. High spawning rates were obtained for Trial 4 and 5, 63% and 83%, respectively. It is noted that the GSI of cohort broodstock that died for both Trials 4 and 5 were 39% and 36% respectively. Other problems remain for future solution. These include occasional low spawning rates, 22-

38% and spawns in which only males spawned, Trials 2, 3, and 6. Single hormone injection was used in all trials except for Trial 6. Among the three successful spawning trials with both spawned male and female, the fecundity ranged from 40,533 eggs (10,133 egg/g BW) to 69,440 eggs (40,000 egg/g BW female) and the fertilization rates were from 18% to 45%. The average hatching rate was 18%. Thus, spawning induction by sGnRHa revealed a better technique that could be used in practical spawning induction of opihi. More attention must be paid to the ripeness level of broodstock.

**Table 6.16.** Spawning trials using hormone sGnRHa.

Trial	n	No. of spawn	% spawn	Total eggs produced	Fertilization rate (%)	Hatching rate (%)
Control (01/01/13)	5	None	-	-	-	-
1 (01/01/13)	8	3 (2F, 1M)	38	69,440	ND	17±1.7
2 (01/10/13)	8	3 (M)	38	-	-	-
3 (01/10/13)	8	2 (M)	25	-	-	-
4 (12/14/13)	8	5 (3F, 2M)	63	200,000	45±4.5	23±2.0
5 (12/14/13)	6	5 (2F, 3M)	83	40,533	18±2.5	14±2.6
6 (12/03/13)	9	2 (M)	22	-	-	-

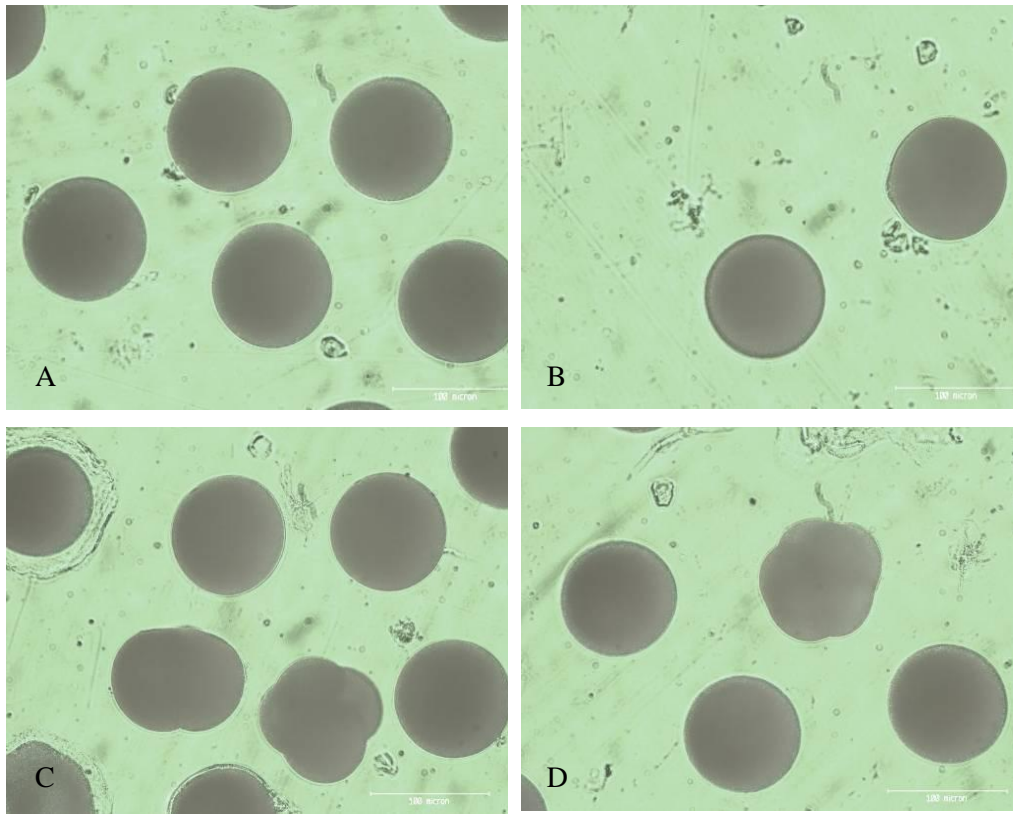
Notes; F: female; M: male.

Embryonic and larval development. Table 6.17 shows the stages and time required for each distinct stage of larval development of opihi at 22°C. Eighteen distinct stages of opihi larval development were observed in this study. The average diameter of spawned eggs (prior to be fertilized) was 111±5.64µm (Fig. 6.12A). Eggs size increased up to 112±7.8 µm after

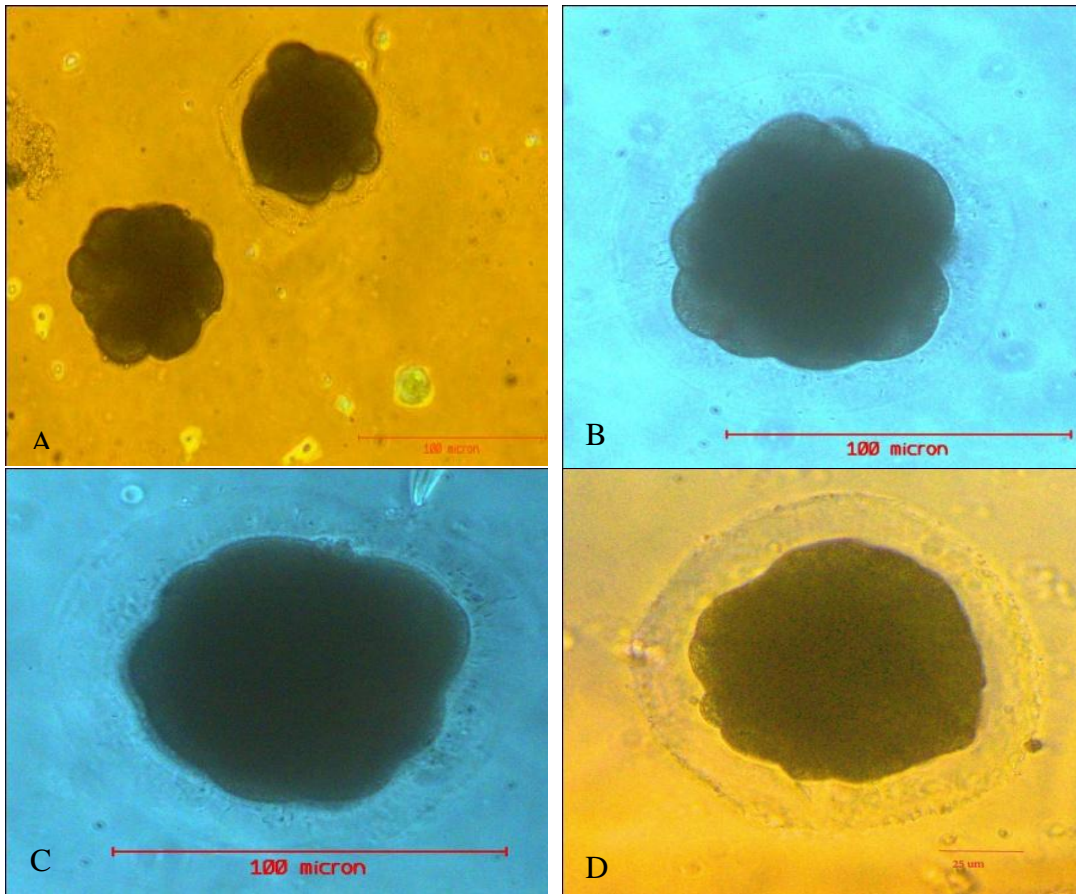
fertilization and sank to the bottom. The extrusion of the first polar body in about 30-45 min after spawning was considered as fertilized eggs (Fig. 6.12B). The two-cells stage, as called stage 3, was reached within 2 h (Fig. 6.12C). Protrochophore stage with cilia appeared in about 10 h post-fertilization (Fig. 6.13C ). Larvae were commenced hatching out at 12-14 h. The length and width of free swimming larvae were  $85.5 \pm 9.5 \mu\text{m}$  and  $79.6 \pm 7.9 \mu\text{m}$ , respectively. The larvae swam by rotating around by cilia from the bottom to the water surface. Larvae continued to develop velum from cilia and apical region became flat for shell formation in about 18-20 hr (Fig. 6.14 and Fig. 6.15).

**Table 6.17.** Embryonic and larval development of opihi *C. sandwicensis* at (22°C).

Sequent stage	Embryo, larval development stage	Time (h)
1	Fertilization	0.00
2	Discharge of first polar	0.30-0.45
3	First cleavage (2 cells)	1.00-1.30
4	Second cleavage (4 cells)	2.00-2.30
5	Third cleavage (8 cells)	3.00-3.30
6	Morula	3.30-4.00
7	Blastula	4.00-4.30
8	Gastrula	4.30-5.00
9	Appearance of cilia forming prototrochal	8.00-10.00
10	Trochophore ready to hatch out	10.30-11.30
11	Trochophore free swimming larvae	12.00-14.00
12	Continue extended cilia	13.30-14.30
13	Completion of griddle and cilia develop	14.30-16.00
14	Larval shell formation	14.30-16.00
15	Advance larvae shell formation	16.30-18.00
16	Exhibiting flat apical from larval shell and complete developed velum and cilia	18.00-20.00
17	Eye spot	20.00-21.30
18	Completed muscle formation	21.30-24.0

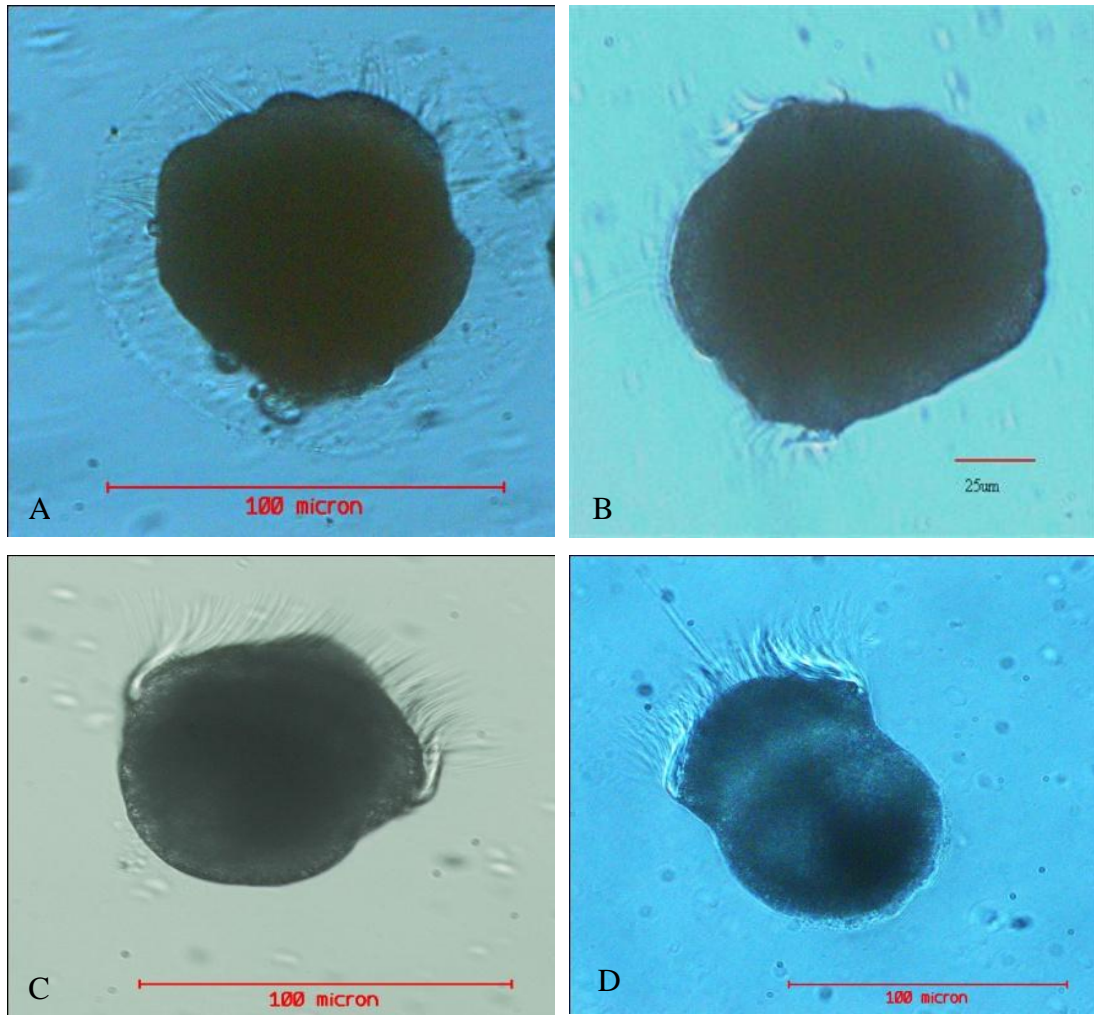


**Figure 6.12.** Embryonic development stage of opihi *C. sandwicensis*. A, spawned egg, and stage 1 spermatozooids. B, stage 2, discharge of polar body. C, stage 3, first cleavage (2 cells) and stage 4, second cleavage (4 cells). D, stage 5, third cleavage (8 cells).

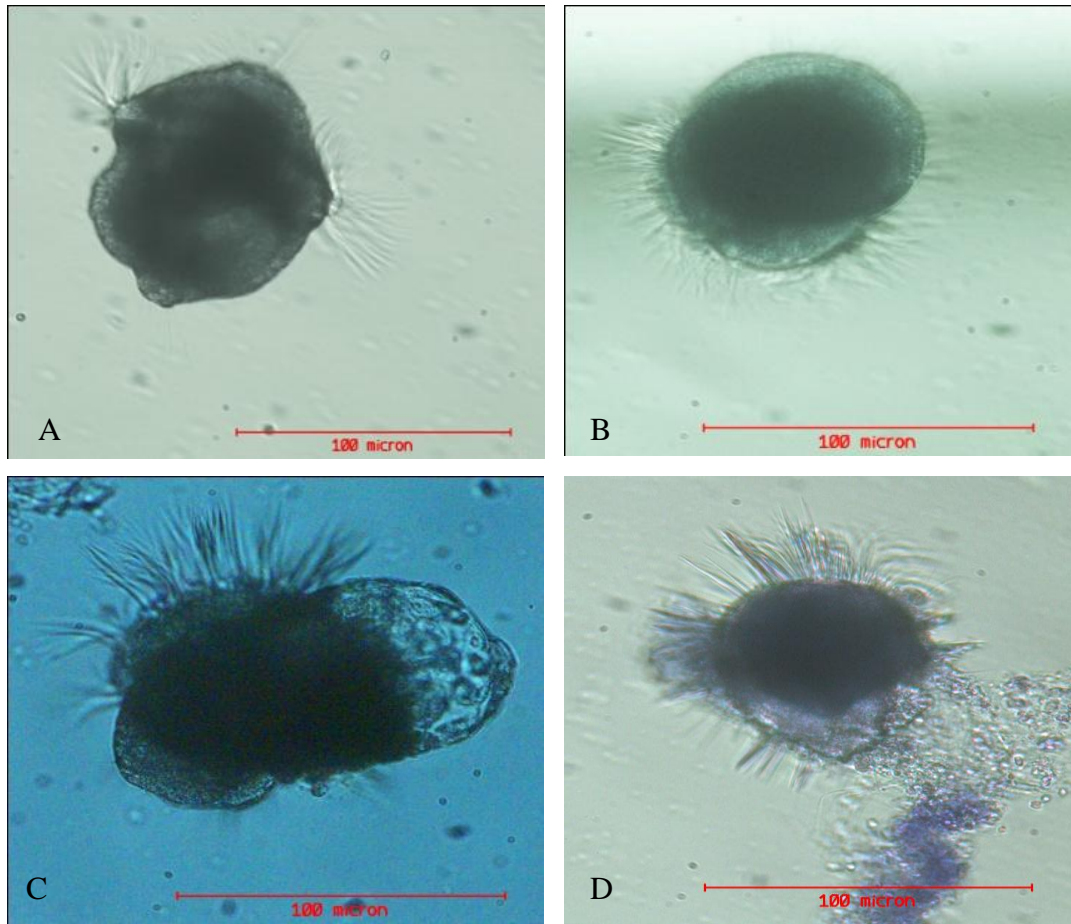


**Figure 6.13.** Embryonic development stage of opihi *C. sandwicensis*.

A, Stage 6-7 morula and blastula. B, stage 8, gastrula. C, D, stage 9, appearance of cilia forming the prototrochal.



**Figure 6.14.** Embryonic and larval development stage of opihi *C. sandwicensis*.  
 A, Stage 10, trochophore larvae ready for hatch out. B, stage 11, trochophore larvae free swimming. C, stage 12, trochophore free swimming larvae with extend cilia. D, stage 13, complete griddle, cilia develop and apical;



**Figure 6.15.** Larval development stage of opihi *C. sandwicensis*.

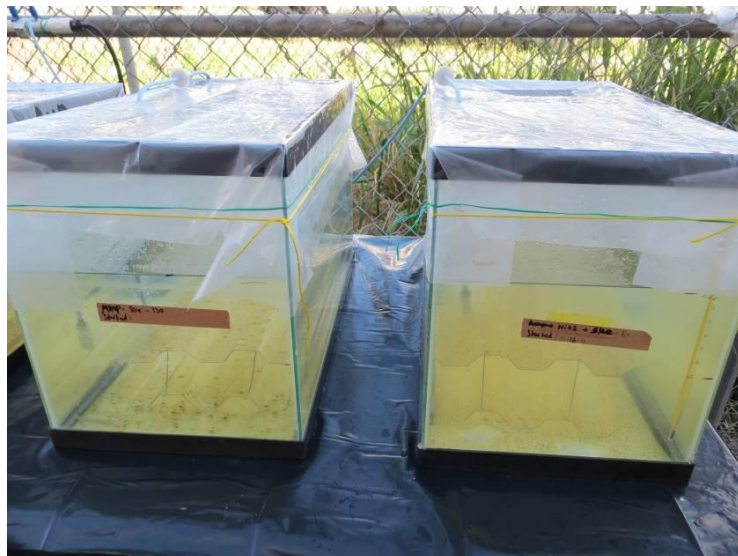
A, stage 14, early larvae shell formation. B, stage 15 and 16, veliger larvae exhibiting flat apical from larval shell and complete developed velum and cilia. C, stage 17, appearance of eye spot. D, stage 18, appearance of muscle attached.

Larval settlement trials. Growth of benthic diatoms and pelagic algae are shown in the Table 6.18. The cell density growth on plate-substrate and pelagic algae in the water column were estimated at Day 5. The larval settlement was started on the day 7. This indicated that the benthic diatoms and pelagic algae could grow a bit higher in cell density than the presented data.

**Table 6.18.** Benthic diatom and pelagic algae growth on plate-substrate and water column at Day 5.

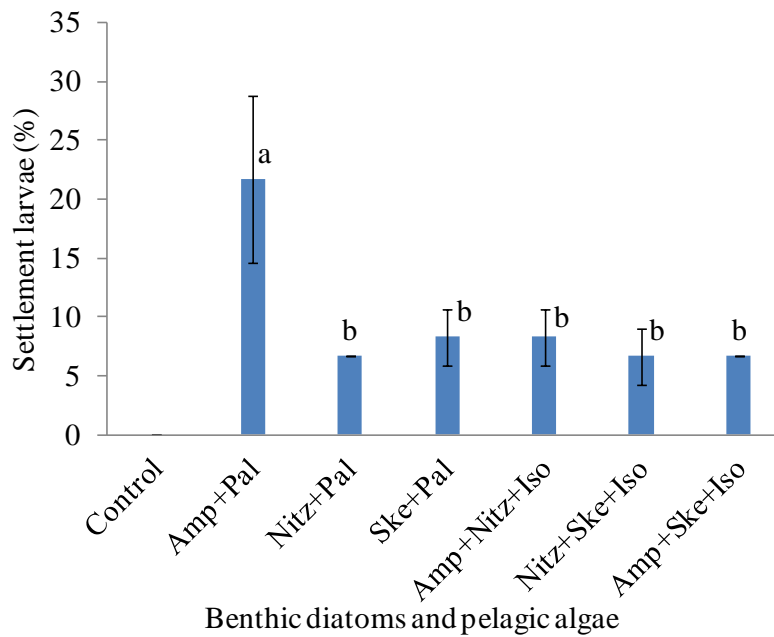
Aquarium	Cell diatom density on plate-substrate (cells/cm <sup>2</sup> ) + pelagic algae/mL	
	Starting	Day 5
<i>Amphora + Pavlova</i>	1,300+18 X 10 <sup>3</sup>	61 X 10 <sup>5</sup> + 114 X 10 <sup>4</sup>
<i>Nitzschia + Pavlova</i>	2,600 + “	58 X 10 <sup>5</sup> + “
<i>Skeletonema + Pavlova</i>	3,000 + “	93 X 10 <sup>5</sup> + “
<i>Amphora, Nitzschia + Isochrysis</i>	2,600 + “	112 X 10 <sup>5</sup> + 138 X 10 <sup>4</sup>
<i>Nitzschia, Skeletonema+ Isochrysis</i>	2,600 + “	89 X 10 <sup>5</sup> + “
<i>Amphora, Skeletonema+ Isochrysis</i>	3,800 + “	93 X 10 <sup>5</sup> + “

The benthic diatoms grew with a thin film on the substrate. Pelagic algae grew to a slight brown color (Fig. 6.16).



**Figure 6.16.** Benthic diatoms and pelagic algae growth on settlement substrate and in the water column after 5 days.

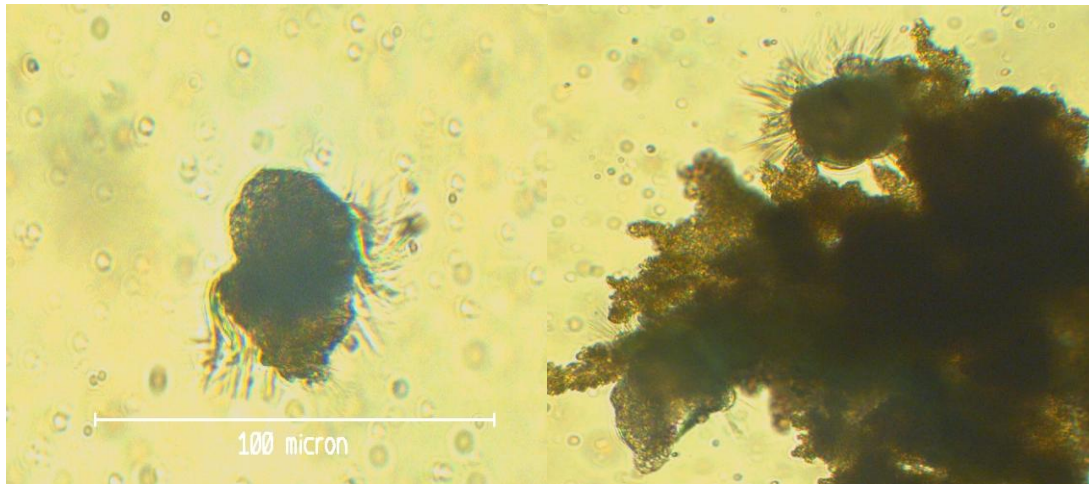
Experimental settlement of larvae on different combinations of diatom and pelagic algae were approached for 9 days in this study. We considered this was our preliminary study because so few animals were studied and all died in a few days. We were only able to collect the settlement of larvae up to the day 6 and the results are presented in the Figure 6.17. There was a significant ( $P = 0.025$ ) difference in larval settlement and metamorphosis over six days period on different diatoms and pelagic algae combination. The highest ( $21.7 \pm 7.07\%$ ) larvae survival after settlement was obtained in beakers that containing *Amphora* and *Palova*. Diatom *Nitzschia* seemed not to be preferred by opihi larvae because the observation noticed that high mortalities occurred from day 4 to day 6 in any beakers that contained *Nitzschia*. Pelagic algal *Palova* may be preferred over *Isocyrisis*. Among the surviving larvae, all of them settled after three days and fed on diatoms. A 30% water change removed dead larvae. The survivors were counted and confirmed under microscope (Fig. 6.17). No survivors were found in the third day of the control beakers without any benthic diatoms and pelagic algae. The size of post-larvae was not measured but it seemed that they were similar size of settled larvae among the diatom diets up to day 6. They were seen until day 9 and thereafter we could not see them just by looking through the beaker due to diatom overgrowth.



**Figure 6.17.** Metamorphosed larvae on different combinations of benthic diatoms and pelagic algae over 6 days period. Data are presented as Mean±SD, n=2 replicate, <sup>a,b</sup>the same letters indicate no significant difference.

Water exchange may not be a problem that washed out larvae, because it was performed very carefully on Day 3 when most of the survived larvae have already settled on the bottom. Water (30%) on top the upper part of the beaker was decanted and new clean seawater containing F/2 medium was refilled slowly into the experimental beakers. Measurement was done carefully by gently pipetting a diatom particle (Fig. 6.18), where settled larvae were located and placed in a petri dish. At this time they have not attached tightly into the substrates yet. These settled larvae were returned to experimental beaker after measurement was done.

Figure 6.18 shows a difference between a free swimming larva and settled larva beginning to undergo metamorphosis after settlement in Day 4. Animals appeared to attach via a muscle attached to diatom particle. Shell length expanded. Cilia were beating.



**Figure 6.18.** Free swimming larvae day 2 (left) and settled larvae fed on diatom in day 4 (right).

None of the settled larvae were seen in the outdoor aquaria after three days as compared to beakers experiment. Only a few settled larvae were seen on day 9 of aquaria containing *Amphora* + *Palova*, and *Nitzchia* + *Palova*. It noticed that the larvae was placed into the aquaria at night time after temperature acclimation, but the temperature raised again from 22°C in the morning up to 28°C in afternoon (the first day of larvae in the settlement tank). The average temperature was 22±0.5°C in the morning and 26±2.0°C in the afternoon. The average dissolved oxygen was maintained at 6.71±0.42 ppm in all aquaria.

## DISCUSSION

In this chapter, our first objective to examine the spawning season of yellow foot opihi *C. sandwicensis* based on the examination of GSI with histological analysis of collected animals monthly. Collecting opihi is restricted by law. The current law that regulates the harvest size for all *Cellana* is 1.25 inches (approximately 3.2 cm) in shell length because it is believed that the animals attain the reproductive stage at this size and only animals that have had a chance to spawn would be subjected to harvest. In our study, the smallest size of animals was 2.86±0.48 cm shell length. These animals were collected in error while collectors were under water. Kay and Magruder (1977) reported that *C. sandwicensis* attained reproductive stage at shell length of 2.3 cm to 2.5 cm or larger. We also noticed that *C. sandwicensis* would attain sexual maturity

size at even smaller than 2.3 cm, in about 1.5-2.0 cm. Thus, the collecting law is consistent with the reproduction science.

The non-reproductive season for yellow foot opihi was proposed to occur from the mid-spring to the summer time. The GSI data ( $12.9 \pm 5.41\%$ ) for February 2012 and following months up to September 2012 were low and remained at the same level. They were significantly lower than other months during the spawning season. The GSI for male ( $11.7 \pm 4.04\%$ ) and female ( $14.4 \pm 4.60\%$ ) was also significantly different with others during the spawning season. These GSI levels are defined as the baseline points and they are used as indicators for further comparison with others. Histology observations for female's gonad in these months were the same as May 2012 (Fig. 6.5A), the oocyte-cell membrane was not clear, and there were a few stage I oocytes. This was true for spermatids as well. The testis was less viscous and it did not stain darkly. In addition, when the GSI was lower than 10%, sex was not identifiable in some cases. This was most frequently seen during March to September of the year. This period could also be called the reproductive resting phase.

This is followed by a final maturation phase which occurs in October. GSI for October ( $18.7 \pm 7.77\%$ ) was significantly higher than the baseline GSI during non-reproductive season. GSI for male ( $23.8 \pm 7.78\%$ ) was significant higher than most the baseline levels during the non spawning season, but GSI for female ( $16.1 \pm 6.86\%$ ) did not differ significantly with non spawners during the non spawning season. The GSI for females may not be indicative of ripeness because a wide range of individual GSI of those animals that were collected in month. It seemed that the gross weight of ovaries was not very changeable as microanatomy as seen by histology. A histology photo for October is shown in the Figure 6.5B. Several oocytes were in the late development stages. No histology analysis was examined for males in October. These data indicate that the gonad of both male and female were in development progress, preparation for a new spawning season and the females were in final maturation in spite of lack of effects on GSI.

The winding down phase occurred in February, after the spawning period. The GSI data for February 2012 ( $9.05 \pm 3.65\%$ ) was winding down to the baseline and it was significantly lower than ripe samples during the spawning season, and it did not differ significantly compared to GSIs during the reproductive resting phase. The GSI for separated males ( $9.16 \pm 4.09\%$ ) and females ( $8.65 \pm 4.14\%$ ) were also winding down and were not significantly different from resting

phase values. Histology photos for February of female and male are shown in Fig. 6.5 E,F. Most of the oocytes were deformed in shape, consistent with reabsorption, and several oocyte stages were present. Spermatozoa tested did not stain dark blue and were mostly pink in color. Thus the conclusion of the spawning season seems to be characterized by GSI values near baseline and histology winding down for females and at baseline formats.

The GSI and histology studies suggest the spawning season of yellow foot opihi commenced in November. The GSI for November was high and significantly higher than those of baseline GSIs during reproductive resting period. Similarly, the GSI for male and female separately were also much higher than the baseline points. Histology photos for the gonad of female and male in November are shown in the Figure 6.5C, D. Most of oocytes appeared mature, large with similar sizes ( $>100\ \mu\text{m}$ ), and showed a round shaped. Egg membranes were distinct and separated the eggs from the rest of the ovary. The testis appeared dense and stained dark blue. They took up hematoxylin stain.

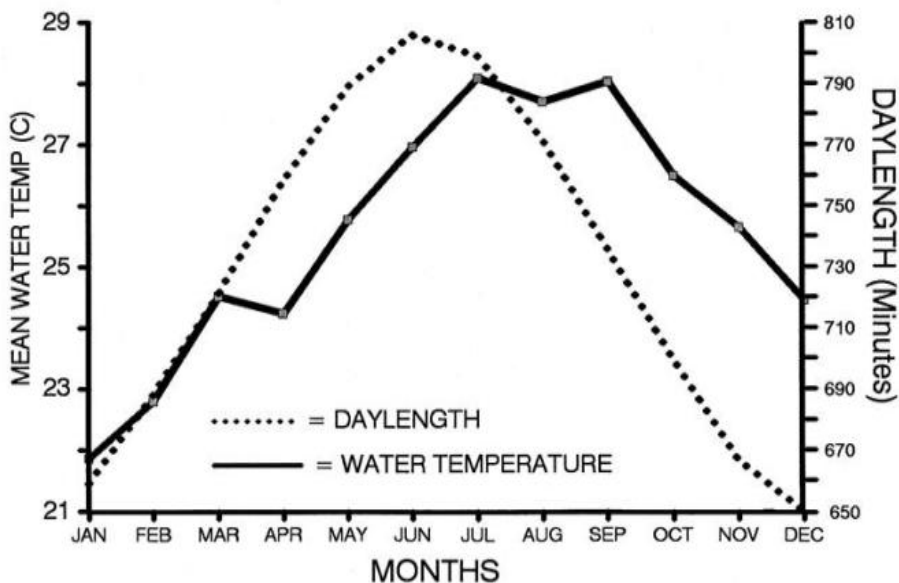
The GSI data for December 2011 was an outlier and could mean that the group of animals sampled had spawned. The GSI data for December 2012 was consistent with the spawning season and higher than others during non spawning season. The separated male GSI was also significantly higher than the baseline GSIs during the non-breeding season and the GSI for females was higher but overlapping with others during non spawning season. This overlap was contradicted by the histology photo for December 2012 of the mature female which was the same as November 2012. Oocytes were round, had distinguishable cell-membranes. Spermatozoa were condensed in the male's gonad. Thus November and December seem like a spawning season.

The GSI value for January 2012 was significantly higher than the baseline GSIs during non spawning season. The GSI for males and females was higher than in non spawning seasons though there was some overlap for males. The histology for females showed mature ovaries, but for males testes were questionably mature. The data suggest that a portion of male opihi population might have been spent or that the male spawning season was winding down.

In conclusion, the results of our study should have concluded that the natural spawning season of the yellow foot opihi would occur from November to December and could be extended to January. The numbers of spawnings during the season are not known and our data might be tainted by spawning within season.

Non-spawning season for yellow foot opihi would occur from late February to early September and gametogenesis development processes was proposed to occur in late September to October, preparation for another spawning cycle. Our present data do not support off-season spawning because we could find evidence for spawning during the off season in our GSI or histology data (Corpuz 1983).

One of the important aspects for the aquaculture of opihi in our study was to mature animals in the laboratory. In our hands photoperiod is also important and the maturation may be impossible if it wasn't for the proper photoperiod, even the maturation diet is properly match or mismatch with nutrient needed. This was proved in our initial maturation trial, none of broodstock matured when animals were fed with the growout diet over three month period (May-August 2012). Besides a poor ARA/EPA ratio (0.045) in the diet, photoperiod was wrong because the experiment was conducted before the final maturation season of opihi. Photoperiod has also been reported to be influential on reproductive cycles of many marine invertebrates (Giese 1959, Giese and Pearse 1974, Maclintock and Watts 1990). A series experiments in laboratory showed that when sea urchin *Eucidaris tribuloides* were held under longer daylength (15L:9D), gametogenesis was delayed over the entire year. The animals that were held under short days (9L:15D) produced mature gonad throughout the year (Maclintock and Watts 1990). Corpuz (1983) found that the reproductive resting phase of *C. exarata* coincided with daylength above 13hr from May to Aug, suggesting that a higher 13 hr daylength could inhibit gametogenesis of opihi. In fact, the Figure 6.19 showed that May to August period which has the greatest daylength (greater than 13 hr). Thus, it appears that the photoperiod has had contributed to the failure in maturation of opihi in this initial maturation trial.



**Figure 6.19.** Temporal changes in water temperature and daylength in Hawaii (provided by Dr. Clyde Tamaru, Windward Community College, Aquaculture Center).

Perhaps the effect of photoperiod may be seen more clearly in the following maturation trials when the experiment was run before and during the spawning season. The improvement of GSI of animals even they were fed with incorrect ARA diets for 45 days (Sep. 23<sup>th</sup>-Nov. 05<sup>th</sup>. 2012), suggesting that the photoperiod might facilitate the gametogenesis process of opihi. The Figure 6.19 showed that the daylength is decreased up to about 10 hr, this period may show to be a favorable time for reproductive development processes. The gonad of animals fed with these incorrect ARA diets were improved slightly (8.13-11.0%) compared to the control group (5.94%). These results reveal that the presence of ARA, even at low levels, in collaboration with a favorable environment would also enhance the proliferation process of opihi.

Correctly proportioned ARA/EPA diets were more effective in enhancing the maturation process for opihi in the second phase of the experiment when photoperiod was during the spawning season. The gonad of animals reached to the final maturity level of about 24% which was examined to be the lowest maturity for female GSI. Histology photos for females fed with these diets demonstrated that most of the oocytes in maturity stages (Fig. 6.9D,E). This indicates that an ARA/EPA (0.70) performed well to enhance the reproductive process of opihi during the

corrected photoperiod. Similar maturation among animals that were fed with the low and high ARA levels indicates that the different ARA levels did not affect the maturation process. The biochemistry suggests that the ratio of ARA/EPA is important.

We did not attempt to induce spawning with these experimental animals because there were only a few animals left after animals were sacrificed for GSI determination for the 75 days sampled. This was a mistake. The data suggested that these animals might have been induced to spawn soon after the 75 days. The reproductive performance was even worse when the animals were fed with correct diet but held under a wrong photoperiod. The GSI was winding down after 95 days for both low ( $10.8 \pm 4.47\%$ ) and high ARA ( $15.5 \pm 5.47\%$ ) diets as compared to GSI of a 75 days measurement. Cross-sections of the ovary confirmed that most of the mature oocytes disappeared and the appearance of several stages of oocytes in the ovary indicates the animals may have undergone natural reabsorption. Nevertheless, our study supports the hypothesis of an appropriate ARA/EPA ratio of 0.70 in the maturation diet would induce final maturation of opihi. We believe that this is our first study on the effect of ARA/EPA on maturation of opihi and gastropods as well.

Similar to the pathway as ARA/EPA, another way of accomplishing the final maturation for opihi would be using sGnRH $\alpha$  injections. The experiment was also done during the natural spawning photoperiod. The sGnRH $\alpha$  stimulated gonad development and final maturation in opihi in 5 weeks when they injected at 7 day intervals at low concentration 250 ng/g BW. The GSI increased significantly from the third week of injection compared to control, which did not show gonadal development. This shows that GnRH also involved in regulating reproductive development in opihi. The mammalian GnRH analogue was known to stimulate maturation and induced spawning in abalone (Nuurai *et al.*, 2010). Joosse (1979) noted that the responses of molluscan to environmental cues are controlled by hormones and the principal sources of hormones within molluscan nervous system are neurosecretory cells. Our results suggest that diatom blooms may be the environment cues. GnRH could stimulate reproductive process by acting directly on the gonad in opihi. Nuurai *et al.* (2010) reported that GnRH like peptide was found in abalone's gonad. This process would be also facilitated by the reproductive photoperiod and/or the right photoperiod would stimulate the increased secretion of LH and FSH that enhances the reproductive process in opihi.

Spawning induction by using hydrogen peroxide was disappointing. Our initial range finding spawning trials proved that most of animals died at  $1.49 \times 10^{-2}\%$  and  $1.80 \times 10^{-2}\%$  in 24 hr after exposure to this level. These results highlighted the non-specific toxic effect of the chemical. Corpuz (1983) reported that all animals were dead eventually at the same level, but this level induced 10-15% spawning. This could probably due to the instability of  $H_2O_2$ . Our  $H_2O_2$  was fresher and we ordered before use. No mortality occurred in 24 hr after spawning at  $0.6 \times 10^{-2}\%$ . This led to the thought that  $0.6 \times 10^{-2}\%$  may be safe, but in the last trial at this concentration all animals died within a week after exposure to  $H_2O_2$ . The opihi may have released gametes because they thought they were dying. This is a well known phenomenon among fruit trees that are sometimes even sprayed with herbicide to get them to fruit. Under the microscope we found that a high percentage of immature eggs with different sizes, these eggs were not successfully fertilized. This concluded that hydrogen peroxide is not a practical method.

Induction of spawning by using sGnRHa is an applicable technique and was the most practical method. There were no mortalities after injection of sGnRHa, and 100% animals survived after spawning. However, it is noticed that spawning induction of opihi by GnRH is effective only on ripe opihi. A low spawning rate (25.0-37.5 %) in GnRH trials could probably due to wild broodstock that were not fully mature before being induced. A limitation of this method is that we were not able to examine exactly the maturity of animals without sacrificing them. Vitellogenesis is a complicated process involves many stages. Current experiences based on the level of GSI and egg size of dead animals of the same cohort before being injected supported this. For example, in the trial 4 and 5 the average GSI of dead animals that were collected in the same day was 39.5% and 36%. A dose of 1,000 ng/g BW was given and most of broodstock (63-83%) spawned. The low spawning rate was obtained in the trial 6 when the GSI of male (about 30.5%) and female (24.8%) was low. Although two injections were applied but there was only 43% of animals spawning and they all were males. This indicates that the females were not fully mature before being induced by hormone, and time interval between the priming dose and the resolving dose needed to be longer. Our final maturation results suggest 3 injections at one week intervals. This would bring all animals to final maturation. Therefore, broodstock with a higher GSI (greater than 30% and/or close to 40%) would give us a better prediction to decide at which dose animals should be administered. Nevertheless, it is shown

that how disadvantages of collecting broodstock from the wild prior to spawning are that their maturity is not as easy to determine as might be guessed and they may not spawn so efficiency. This is a common issue also seen in abalone. Spawning of temperate abalone specie *H. fulgens*, was found to be difficult when animal were collected directly from the wild (Hahn 1989). Therefore, mature animal in the laboratory would avoid these problems. They can be natural spawn in captive conditions.

A completed embryonic and larval development processes of *C. sandwicensis* was observed in this study. Embryonic development occurred rapidly by morphologies change from embryo to the formation of trochophore and subsequent veliger stage. The embryonic processes develop through many stages, each stage has a particular distinct characteristic such as first cleavage, second, third cleavage, blastula, gastrula, and so on. We found 18 distinct embryonic development stages. Duration for each particular stage was different. The first polar body was useful indication of successful fertilization but we often miss this stage because it happened in short period of time (30 min) after fertilization. The first cleavage (2 cells) is additional evidence that we can examine fertilization successful or not. The embryonic development was not a consistent process there were always several embryo stages were observed at a certain time. Trochophore free swimming larvae were observed in 10-12 hr and were followed by subsequent veliger stage 12-14 hr after fertilization at 22°C. The rate of embryonic development in this study was consistent with that obtained from Corpuz (1983). However, we documented detail characteristics in each particular stage in associated with timing.

Our attempt was trying to test the survival and possible metamorphosis on different combinations of benthic diatoms (*Amphora*, *Nitzschia* and *Skeletonema*) and pelagic algae (*Palova* and *Isochrysis*). The data are only valid for speculation due to small number of animals and short survival time (died by Day 9). We speculate that a combination of *Amphora* and *Palova* yield the highest survival. This is a combination of pelagic algae that can be eaten when the larvae are pelagic and benthic algae after they settle. In control experiments no larvae survive in beakers without algae. The benthic diatom *Amphora* was one of the predominant algae in the stomach content of adult opihi in a prior study. However, this benthic diatom alone may be not enough nutrients for settled larvae. As stated earlier, a mixture of benthic diatoms produced the best growth and survival for settled abalone larvae (Gapasin and Polohan 2005). It

suggests that we should have more appropriate benthic diatoms growth on plates for settlement trials beside *Amphora*.

Possibly, the overgrowth on benthic diatoms on plate-substrates must be taken into account. The cell density on the plates prior to the experiment in our study ( $61 \times 10^5$ - $112 \times 10^5$  cell/cm<sup>2</sup>) was about 20-60 times higher than other study for the settlement of abalone larvae ( $1 \times 10^5$ - $6 \times 10^5$  cell/cm<sup>2</sup>, Gapasin and Polohan 2005). For practical purposes, it was difficult to see settled larvae in beakers after Day 4 due to diatom overgrowth. Because a 24 hr fluorescent light was provided for the first three days after the larvae have settled. It was happened in the indoor aquaria systems. Per communication with Cecilia Viljoen (Big Island Abalone), a 80% shade cloth cover cultures was used after the settlement of abalone larvae on substrates for the first 7 days and combination with a slow flow through seawater (5 mL per min). This could probably be an option for opihi, we should have tried it in a few days after settlement.

All larvae died by Day 9. It is impossible to know why but one speculation could be nutrient deficiencies among the diatoms we used and low temperature (22°C) in air conditions in the laboratory is not good either. Diatoms were not being held in the sun. In previous work benthic diatoms seemed to be a good natural food source for holding opihi until feeding trials were done but later we found that diatoms did not support good growth. The same can be said of macroalgae versus artificial feeds for abalone. Much more efforts are needed to complete the life cycle of opihi for future aquaculture without relying on wild stocks.

## LITERATURES CITED

- Ako, H. 1995. Algae in the Aquaculture of Marine Fishes and Shrimps. Sustainable Aquaculture 95, proceedings of the Pacific Congress on Marine Science and Technology, PACON, pp. 7-14. Page 38.
- Ako, H. and C. S. Tamaru. 1999. Are feeds for food fish practical for aquarium fish. International Aquafeed 2: 30-36.
- Ako, H. and W. Dominy. 1987. The amino acid requirements of Penaeid shrimp. University of Hawaii Sea Grant Quarterly 9 (4):1-8.
- Alam, Md.S., Teshima, S.I., Koshio, S., Uyan, O. and Ishikawa, M. 2003. Effects of dietary protein and lipid levels on growth and body composition of juvenile Japanese flounder, *Paralichthys olivaceus*, fed diets containing intact proteins or crystalline amino acids. Journal of Applied Aquaculture 14: 115-131.
- Amano, M., S. Moriyama, K. Okubo, N. Amiya, A. Takahashi, and Y. Oka. 2010. Biochemical and Immunohistochemical Analyses of a GnRH-like Peptide in the Neural Ganglia of the Pacific Abalone *Haliotis Discus Hannai* (Gastropoda). Zoological Science 27(8):656-661.
- Baba, Y., H. Matuso, and A.V. Schally. 1971. Structure of the porcine LH- and FSH-releasing hormone. II. Confirmation of the proposed structure by conventional sequential analysis. Biochemical and Biophysical Research Communication 44: 459-463.
- Barkai, R. and C. L. Griffiths. 1987. Consumption, absorption efficiency, respiration and excretion in the South African abalone *Haliotis midae*. South African Journal of Marine Science 5:523-529.
- Bautista-Teruel, M., A. C. Fermin, and S. S. Koshio. 2003. Diet development and evaluation for juvenile abalone, *Haliotis asinina*: animal and plant protein sources. Aquaculture 219: 645-653.
- Bird, C. E. 2011. Morphological and behavioral evidence for adaptive diversification of sympatric Hawaiian limpets (*Cellana* spp.). Integrative and Comparative Biology. 1-8.
- Bird, C. E. 2006. Aspects of community ecology on wave-exposed rocky Hawaiian coasts. Doctoral dissertation, University of Hawai'i at Mānoa, Honolulu.
- Bird, C. E., B. S. Holland, B. W. Bowen, and R. J. Toonen. 2007. Contrasting phylogeography in three endemic Hawaiian limpets (*Cellana* spp.) with similar life histories. Molecular Ecology 16: 3173-3186.
- Branch, G. M. 1981. The biology of limpets: physical factors, energy flow, and ecological interactions. Oceanogr. Mar. Biol. Ann. Rev. 19 p 235-380.
- Britz, P. J. 1996. Effect of dietary protein level on growth performance of South African abalone. *Haliotis midae*, fed fishmeal-based semi-purified diets. Aquaculture 140:55-61.
- Britz, P.J. and T. Hecht. 1997. Effect of dietary protein and energy level on growth and body composition of South African abalone *Haliotis midae*. Aquaculture 156: 195-210.

- Brown, M. R., S. W. Jeffrey, J. K. Volkman, and G. A. Dunstan. 1997. Nutritional properties of microalgae for mariculture. *Aquaculture* 151: 315-331.
- Buhler, D.R. and J.E. Halver. 1991. Nutrition of Salmon fishes. IX. Carbohydrate requirements of Chinook salmon. *Journal of Nutrition* 74:307-318.
- Capinpin Jr, E. C. 2007. Feeding, growth, and survival of post-larval abalone *Haliotis asinine* on different benthic diatoms. *Science Diliman* 19 (2): 49-59.
- Cho, S. H. 2010. Effect of fishmeal substitution with various animal and/or plant protein sources in the diet of the abalone *Haliotis discus hannai* Ino. *Aquaculture Research* 41:587-593
- Cho, S. H., J. Park, and J. H. Yoo. 2008. Effect of casein substitution with fishmeal, soybean meal and crustacean meal in the diet of the abalone *Haliotis discus hannai* Ino. *Aquaculture Nutrition* 14: 61-66.
- Coote, T. A., P. W. Hone, R. W. Van Barneveld, and G.T. Maguire. 2000. Optimal protein level in a semipurified diet for juvenile greenlip *Haliotis laevigata*. *Aquaculture Nutrition* 6:213-220.
- Corpuz, G. C. 1981. Laboratory culture of *Cellana exarata* Reeve (Gastropoda: Prosobranchia, Patellidae). *Aquaculture* 24:219-231.
- Corpuz, G. C. 1983. Life history traits influencing vertical zonation in the Hawaiian intertidal species of *Cellana*. Doctoral thesis. University of Hawaii, Manoa, Hawaii, USA.
- Cox, K. W. 1962. Californian abalones, Family Haliotidae. *California Fish and Game Fisheries Bulletin*, 118. 133p
- Dabrowski, K. and H. Guderley. 2002. Intermediary metabolism in fish. In. *Fish nutrition*. The third edition. Halver and Hardy (Edit). Academic Express, London. pp 309-332.
- Daume, S., A. Krsinich, S. Farrell, and M. Gervis. 2000. Settlement, early growth and survival of *Haliotis rubra* in response to different algal species. *Journal of Applied Phycology* 12: 479-488.
- Dworjanyn, S. A., I. Pirozzi, and W. Liu. 2007. The effect of the addition of algae feeding stimulants to artificial diets for the sea urchin *Tripneustes gratilla*. *Aquaculture* 273: 624-633.
- Felix, N. and M. Sudharsan. 2004. Effect of glycine betaine, a feed attractant affecting growth and feed conversion of juvenile freshwater prawn *Macrobrachium rosenbergii*. *Aquaculture Nutrition* 10:193-197.
- Fleming, A. E., R. Van Bameveld, and P. W. Hone. 1996. The development of artificial diets for abalone: A review and future directions. *Aquaculture* 140:5-53.
- Furuita, H., T. Yamamoto, T. Shima, N. Suzuki, and T. Takeuchi. 2003. Effect of arachidonic acid levels in broodstock diet on larval and egg quality of Japanese flounder *Paralichthys olivaceus*. *Aquaculture* 22: 725-735.
- Gapasin, R. S. J. and B. B. Polohan. 2005. Response of the tropical abalone, *Haliotis asinina*, larvae on combinations of attachment cues. *Hydrobiologia* 548:301-306.

- Giese, A. C. 1959. Comparative physiology: animal reproductive cycles of marine invertebrates. *Annual Review of Physiology* 547-57.
- Giese, A. C. and J.S. Pearse. 1974. Introduction: general principles. In: Giese, A. C., Pearse, J. S. (edited.) *Reproduction of marine invertebrates*, Vol. 1. Academic Press, New York, pp. 1-49.
- Gilmartin, M. and N, Revelante. 1974. The 'island mass' effect on the phytoplankton and primary production of Hawaiian Islands. *Journal of Experimental Marine Biology Ecology* 16: 181-204
- Goezt, F.W. 1983. Hormonal control oocyte final maturation and ovulation of fishes. In: W.S. Ho and D.J. Randall (editors), *Fish Physiology*, Vol. 9B. Academic Press, New York, NY pp.117-169.
- Gomez-Montes, L., Z. Garcia-Esquivel, L. R. D'Abramom, A. Shimada, C, Vasquez-Pelaez, and M. T. Viana. 2003. Effect of dietary protein:ratio on intake, growth and metabolism of juvenile green abalone *Haliotis fulgens*. *Aquaculture* 220:769-780.
- Gordon, N., A. Neori, M. Shpigel, M. J. Lee, and S. Harpaz. 2006. Effect of diatom diets on growth and survival of the abalone *Haliotis discus hannai* postlarvae. *Aquaculture* 252: 225-233.
- Gorfine, H. K. 1991. An Artificial Diet for Hatchery-reared Abalone *Haliotis rubra*. Marine Science Laboratories, Internal Rep. No. 190, Queenscliff, Australia.
- Guillard, R. R. L. and J. H. Ryther. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. *Canadian Journal of Microbiology* 8: 229-239.
- Hahn, K. O. 1989. Artificial induction of condition (gonad maturation). In: *Handbook of culture of abalone and other marine gastropods* (Hahn, K.O edited). CRC Press, Inc. Boca Raton, Florida. pp 41-52.
- Hahn, K. O. 1994. The neurosecretory staining in the cerebral ganglia of the Japanese abalone (Ezo-awabi), *Haliotis discus hannai*, and its relationship to reproduction. *General and Comparative Endocrinology* 93:295-303
- Handbook on ingredients for aquaculture feeds, edited by Hertrampf and Piedad-Pascual. 2000. Kluwer Academic Publishers, Boston. Pp: 177-240.
- Handy, E. S., E. Handy, and M. K. Pukui. 1991. Native planers in old Hawai'i, their life, lore and environment. Bishop Museum Press, Honolulu, Hawai'i.
- Harada, K. and Y. Akishima. 1985. Feeding attraction activities of proteins, amino acids, lipids and nitrogenous bases for abalone. *Bulletin of Japanese Society of Scientific Fisheries* 5(1): 2051-2058.
- Hemre, G.I., Mommsen, T.P. and Krogdal, A. 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquaculture nutrition* 8: 175-194.
- Hidalgo, M. C., E. Urea, and A. Sanz. 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture*. 170: 267-283.

- Hua, T. N. and H. Ako. 2012. Enabling studies for aquaculture of the Hawaiian Opihi, the Limpet *Cellana*. *World Aquaculture Society* 12: 40-44.
- Hyslop, E. J. 1980. Stomach contents analysis- a review of methods and their applications. *Journal of Fish Biology* 17: 411-429.
- Iacchei, M. 2011. Managing fisheries for future. *Ka Pili Kai* 33:3-5.
- Iacchei, M. 2012. The impact of opihi on intertidal communities. *Ka Pili Kai* 33:3-5.
- Joose, J. 1979. Evolutionary aspects of endocrine system and the hormonal control of reproduction of mollusk. In *Hormones and Evolution*, Vol. I (B Arrlington, E. J. W. edited). Academic Press, New York. pp. 199-157.
- Ju, Z.Y., I. Forster, L. Conquest, W. Dominy, W. C. Kuo, and F. D. Horgen. 2008. Determination of microbial community structures of shrimp floc cultures by biomarkers and analysis of floc amino acid profiles. *Aquaculture Research* 1-16.
- Kawamura, T. 1996. The role of benthic diatoms in the early life stages of the Japanese abalone (*Haliotis discus hannai*). In: Watanabe, Y., Yamashita, Y., Oozeki, Y. (Eds.), *Survival Strategies in Early Life Stages of Marine Resources*. Balkema, Brookfield, pp. 355–367.
- Kay, C. M. and R. B. Emlet. 2002. Laboratory spawning, larval development, and metamorphosis of the limpets *Lottia digitalis* and *Lottia asimi* (Patellogastropoda, Lottoidae). *Invertebrate Biology* 121 (1): 11-24.
- Kay, E. A. and W. Magruder. 1977. *The biology of opihi*. Department of planning and economic development. Honolulu.
- Kay, E. A., Bird, C.E., Holland, B.S., and C. M., Smith. 2006. Growth Rates, Reproductive Cycles, and Population Genetics of Opihi from the National Parks in the Hawaiian Islands. *Ecology of KALA marine resources-Brown et al.*, 2006.
- Knauer, J., P. J. Britz, and T. Hecht. 1993. The effect of seven binding agents on 24hour water stability of an artificial weaning diet for the South African abalone, *Haliotis midae* (Haliotidae, Gastropoda). *Aquaculture* 115: 327-334.
- Knauer, J., P. J. Britz, and T. Hetcht. 1996. Comparative growth performance and digestive enzyme activity of juvenile South African abalone, *Haliotis midae*, fed on diatoms and a practical diet. *Aquaculture* 140: 75-85.
- Lee, C. S., C. S.Tamaru, C. D. Kelley, and J. E. Banno. 1986b. Induced spawning of milkfish, *Chanos chanos*, by a single application of LHRH-Analogue. *Aquaculture* 58: 87-98.
- Lee, C.S., C. S. Tamaru, J. E. Banno, C. D. Kelley, A. Bocek, and T. A. Wyban. 1986a. Induced maturation and spawning of Milkfish *Chanos chanos* forsskal, by hormone implantation. *Aquaculture* 52: 199-205.
- Lee, R. E. 2008. *Phycology*. Cambridge University Press, 4<sup>th</sup> edition.
- Lee, S. M., S. J. Yun, K.S. Min, and S. K. Yoo. 1998. Evaluation of dietary carbohydrate sources for juvenile abalone *Haliotis discus hannai*. *J. Aquacult.* 11:133-140.
- Lee, C. S., C. S. Tamaru, G.T. Miyamoto, and C. D. Kelley. 1987. Induced spawning of grey mullet (*Mugil cephalus*) by LHRH-a. *Aquaculture* 62: 327-336.

- Livingstone, D.R. and de Zwaan, A. 1983. Carbohydrate metabolism of gastropods. In: The mollusca, Vol. 1. Metabolic Biochemistry and molecular Biomechanics (Hochachke, P.W. Ed.), pp. 177-242. Academic Press, New York.
- Luo, Z., X. Y. Tan, X. J. Liu, and H. Wen. 2011. Effect of dietary betaine level on growth performance and hepatic intermediary metabolism of GIFT strain of Nile tilapia *Oreochromis niloticus* rearing in freshwater. *Aquaculture nutrition* 17:361-367.
- Mai, K., J. P. Mercer, and J. Donlon. 1995. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. IV. Optimum dietary protein level for growth. *Aquaculture* 136: 165-180.
- Matsuo, H., A. Arimura, and A.V Schally. 1971a. Synthesis of the porcine LH- and FSH-releasing hormone by the solid-phase method. *Biochemistry and Biophysical Research Communications* 45: 822-827.
- Matsuo, H., Y. Baba, and R. M. G. Nair. 1971b. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochemistry and Biophysical Research Communications* 43: 1334-1339.
- McCarthy, M., P. Woosnam, and S. C. Culloty. 2008. Histological investigation of the reproductive cycles of limpets *Patella vulgate* and *Patella ulyssiponensis*. *Marine Biology* 153: 871-877.
- McClintock, J. and S. Watts. 1990. The effects of photoperiod on gametogenesis in the tropical sea urchin *Eucidaris tribuloides*. *Journal of Experimental Marine Biology and Ecology*, 139: 175-184.
- Mengqing, L., L. Qing, and A. A. Anders. 2002. Feed attractants and protein quality meal less waste. *FEED MIX* 10(3): 31-34.
- Morse, D. E. 1984. Biochemical and genetic engineering for improved production of abalone and other valuable mollusks. *Aquaculture* 39: 263-282.
- Morse, D. E., H. Duncan, N. Hooker, and A. Morse. 1977. Hydrogen peroxide induces spawning in mollusks, with activation of prostaglandin endoperoxide synthetase, *Science* 196: 298-300.
- Moss, G. A., J. Illingworth, and L. J. Tong. 1995. Comparing two simple methods to induce spawning in the New Zealand abalone (Pauna) *Haliotis iris*. *New Zealand Journal of Marine and Fresh Water Research* 29: 329-333.
- Naidoo, K., M. Gavin, K. Ruck, and J. J. Bolton. 2006. A comparison of various seaweed-based diets and formulated feed on growth rate of abalone in a land-based aquaculture system. *Journal of Applied Phycology* 18: 437-443.
- National Research Council (NRC). 2011. *Nutrient Requirements of Fish*. The National Academy Press, Washington, D.C. Pp: 345-346.
- National Research Council (NRC). 1981. *Nutrient Requirements of coldwater Fishes*, No. 16. The National Academy Press, Washington, D.C.
- National Research Council (NRC). 1993. *Nutrient Requirements of Fish*, pp: 345-346. The National Academy Press, Washington, D.C.

- Nicotri, M. E. 1977. Grazing effects on four marine intertidal herbivores on the microflora. *Ecology* 58: 1020-1032.
- Nuurai P., A. Engsusophon, T. Poomtong, P. Sretarugsa, P. Hanna, P. Sobhon, and C. Wanichanon. 2010. Stimulatory Effects of Egg-Laying Hormone and Gonadotropin-Releasing Hormone on Reproduction of the Tropical Abalone, *Haliotis asinina* Linnaeus, *Journal of shellfish Research* 29 (3):627-635.
- Pukui, M. K. 1983. 'Ōlelo No'eau: Hawaiian proverbs and poetical sayings. Bishop Museum Press, Honolulu, Hawai'i.
- Saboski, E. M. 1976. Physiological ecology of Hawaiian, marine psammolittoral diatoms. PhD Thesis, University of Hawaii at Manoa, Hawaii, USA.
- Sales, J., P. J. Truter, and P. J. Birtz. 2003. Optimum dietary crude protein level for growth in South African abalone (*Haliotis midae* L.). *Aquaculture Nutrition*. 9: 85-89.
- Satpathy, B.B., Mukherjee, D., and Ray, A.K. 2003. Effects of dietary and lipid levels on growth, feed conversion and body composition in rohu, *Labeo rohita* (Hamilton), fingerlings. *Aquaculture nutrition* 9: 17-24.
- Schally, A.V., A. Arimura, and A.J. Kastin. 1971a. Isolation, structural determination and synthesis of hypothalamic LH and FSH releasing hormone. *Res. Reprod.*, 3, 1.
- Schally, A.V., A. Arimura, and Y. Baba. 1971b. Purification and properties of the LH and FSH-releasing hormone from porcine hypothalamus. Program of the 53<sup>rd</sup> meeting of the Endocrine Society, San Francisco, California, June 24–26, Abstract no.55, p. A70.
- Schimz, G. and J. Ecker. 2008. The opposing effect of n-3 and n-6 fatty acids. *Prog. Lipid. Res.* 47:147-155.
- Searcy-Bernal, R., A. E. Salas, R. A. Flores-Aguilar, and P. R. Hinojosa-Rivera. 1992. Simultaneous comparison of methods for settlement and metamorphosis induction in the red abalone *Haliotis rufescens*. *Aquaculture* 105:241-250.
- Sherwood, N. M., D. B. Parker, J. E. McRory, and D. W. Lescheid. 1994. Molecular evolution of growth hormone-releasing hormone and gonadotropin-releasing hormone. In: Sherwood, N.M., Hew, C.L. Eds. , *Fish Physiology. Molecular Endocrinology of Fish*, vol. XIII, Academic Press, New York, pp. 3–66.
- Sherwood, N. M., L. Eiden, M. Brownstein, J. Spiess, J. Reveir, and W. Vale. 1983. Characterization of a teleost gonadotropin-releasing hormone. *Proc. Natl. Acad. Sci. U SA.* 80: 2794-2798.
- Shiau, S.Y. and J.C. Chuang. 1995. Utilization of disaccharides by juvenile tilapia, *Oreochromis niloticus* and *O. aureus*. *Aquaculture* 133: 249-256.
- Steneck, R. S. 1982. A limpet-coralline algae association: adaptation and defenses between a selective herbivore and its prey. *Ecology* 63 (2): 507-522.
- Tamaru, C. S., H. Ako, and C.S. Lee. 1992. Fatty acid and amino acid profiles of spawned eggs of striped mullet, *Mugil cephalus* L. *Aquaculture* 105: 83-94.
- Tamaru, C. S., H. Ako, and R. Panguirigan. 1997. Essential fatty acid profiles of maturation feeds used in freshwater ornamental fish culture. *Hydrobiologia* 358: 265-268.

- Taylor, B. 1997. Protein utilization, hormone treatment and nutrient metabolism as they apply to culture of *Haliotis kamtschatkana*. PhD thesis, the University of British Columbia, Canada.
- Thompson, D. 2011. The Honolulu Magazine. The opihi shellfish story.  
<http://www.honolulumagazine.com/Honolulu-Magazine/November-2011/The-Opihi-Shellfish-Story/>
- Thongrod, S., M. Tamtina, C. Chairatb, and M. Boonyaratpalin. 2003. Lipid to carbohydrate ratio in donkey's ear abalone *Haliotis asinina*, Linne diets. *Aquaculture* 225:165-174.
- Tsai, P. S., T. A. Maldonado, and J. B. Lunden. 2003. Localization of gonadotropin-releasing hormone in the central nervous system and a peripheral chemosensory organ of *Aplysia californica*. *Gen. Comp. Endocrinol.* 130: 20-28.
- Tung, P. H. and S. Y. Shiau. 1993. Carbohydrate utilization versus body size in Tilapia, *Oreochromis nolticus* x *O. aurens*. *Comp. Biochem. Physical.* 104A (3): 583-588.
- Uki, N. 1981. Feeding behavior and experimental populations of abalone *Haliotis discus hanai*. *Bulletin of Tohoku Regional Fisheries Research Laboratory* 43: 53-58.
- Uki, N. and T. Watanabe. 1989. Review of nutritional requirements of abalone (*Haliotis* pp.) and development of more efficient artificial diets. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmiiin Del Proo (Editors), *Abalone of the World. Fisheries, Biology and Culture.* Fishing News Books, Oxford, pp. 504-517.
- Uki, N., A. Kemuyama, and T. Watanabe. 1985. Nutritional evaluation of several protein sources in diets for abalone *Haliotis discus hannai*. *Bulletin of the Japanese Society of Scientific Fisheries* 51:1835-1839.
- Uki, N., and T. Watanabe. 1989. Review of nutritional requirements of abalone (*Haliotis* spp.) and development of more efficient artificial diets. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmiiin Del Pr6. (Editors), *Abalone of the World. Fisheries, Biology and Culture.* Fishing News Books, Oxford, pp. 504-517.
- Uki, N., M. Sugiura. and T. Watanabe. 1986. Requirements of essential fatty acids in the abalone *Halototis discus hannai*. *Nippon Suisan Gakkaishi* 52: 1013-1023 (in Japanese with Abstract in English).
- Volkman, J. K., S.W. Jeffrey, P. D. Nichols., G. I. Rogers, and C. D. Garland. 1989. Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *Journal of Experimental Marine Biology and Ecology* 128: 219-214.
- Wilson, R.P. and W.E. Poe. 1987. Apparent inability if channel catfish to utilize dietary mono- and disaccharides as energy source. *Journal of Nutrition* 17: 280-285.