Chapter

# Reproductive Biology, Seed Production, and Culture of the Hawaiian Limpet *Cellana sandwicensis* (Pease, 1861)

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## Abstract

The purpose of this chapter was to describe the current finding on the development of aquaculture technologies for the Hawaiian limpet *Cellana sandwicensis*, known as "yellow opihi" in Hawaii. Some reproductive biology characteristics of *C. sandwicensis* were reported including spawning season, gonad maturation stages, maturity size, and fecundity. Monthly record of gonadosomatic index (GSI) suggested that the natural spawning season of *C. sandwicensis* occurred from November to January. Attempting studies on seed production have also performed and achieved several important key points such as inducing final maturation by incorporating arachidonic acid (ARA) into the diet and injecting salmon gonadotropin-releasing hormone analog (sGnRHa). Laval metamorphosis and settlement were successfully induced using a combination of algae *Palova* and benthic diatom *Amphora*. Stomach content analysis gave an insight into the palatability factor for further development of artificial feed; later on, the algae *Porphyra* commonly known as Nori was as attractive as a biofilm and was used as a feeding stimulant. Nutritional study on specific nutrient requirements such as protein, carbohydrate, and energy has been conducted and found that dietary 35% protein, 32% carbohydrate, and protein to energy (PE) ratio ranging from 87.2 to 102.9 mg/kcal could be used for the development of commercial feed for limpet *C. sandwicensis*.

**Keywords:** Hawaiian limpet *Cellana sandwicensis*, yellow opihi, spawning season, seed production, nutrient requirement

## 1. Introduction

Limpets are marine gastropods. They distribute at different intertidal zones of most oceans, from the upper littoral to the shallow subtidal on the rocky coasts. They feed by grazing on macroalgae, benthic diatom growing on rocky substrate because they attach themselves to rocks, and/or any substratum using pedal mucus and a muscular "foot," which also enables them to go against dangerous wave action, desiccation, and predator.

*Cellana* genus is a marine gastropod mollusk in the family Nacellidae [1]. This genus distributes in the temperate and tropical Indo-Pacific Oceans, Hawaii,

Australia, and New Zealand. Species are also found around the coasts of Japan, the Red Sea, Madagascar, South Africa, and the subantarctic island. There are more than 58 species of this genus. Among of those, many of them are of high economic value and aquaculture, for example, the two species *Cellana talcosa* and *Cellana sandwicensis* are expensive in Hawaii.

In Hawaii, there are three main endemic Hawaiian limpet species, called "opihi," including black foot or makaiauli (*Cellana exarata*), yellow foot or ālinalina (*C. sandwicensis*), and the largest species, giant limpet or kōʻele (*C. talcosa*) [2]. Natural ecological distribution of Hawaiian limpet is different intertidal zones of habitation on rocky shores. *C. exarata* is commonly found at higher intertidal zones, and *C. sandwicensis* is at low intertidal zones and rarely exposed by tide, whereas *C. talcosa* distributes in deep water [3–6]. These species are herbivorous grazers that feed on benthic microalgae, diatoms that growing hard substrates such as rocky substrates, death coral reef, and so on. They use teeth in their radula to graze on the toughest crustose coralline algae [3]. They are considered as high-value food market and high-potential candidate species for commercial aquaculture. High commercial catch reduced significantly from 150,000 pounds in the 1900s to about 10,000 pounds in 1978 [7]. The scarcity has boost up prices to about $200/gallon with shell on [8].

In addition to important food sources, these Hawaiian limpet species are also culturally important in Hawaiian society. Many people (opihi pickers) were asked to collect these opihi for parties or family gathering with high prices. Besides that limpet's shell also continue to be used as tools for scraping skin off taro plant and sweet potato and grating coconut meat before eating [9] and as decorative elements in jewelry.

The success of any aquaculture species depends on seed production in captivity. Understanding the completion of the life cycle of limpet would make limpet aquaculture sustainable. The first priority is to understand some reproductive characteristics of the limpet species. This would also provide us with better knowledge for breeding limpet in the hatchery. In this chapter, we describe the current finding on reproductive biology, seed production, nutrient requirement, and culture techniques for the Hawaiian limpet *Cellana sandwicensis*.

## 2. Some reproductive characteristics of Hawaiian limpet *C. sandwicensis*

Reproductive characteristics of Hawaiian limpet *C. sandwicensis* have been reported by several studies [10–12]. The main focused reproductive criteria were spawning season, gonad development stage, fecundity, and maturity size.

### 2.1 Spawning season

A total of 266 specimens (**Table 1**) were sampled for a 1-year cycle (November 2011–December 2012) in Hawaii Island, and gonadal somatic index (GSI) was determined. The GSI was calculated according to equation GSI = (GW/BW) × 100, where GW is gonad weight and BW is body weight or soft body tissue. Gonad development stage was also evaluated by using histological examination. The result showed that the highest average GSI of *C. sandwicensis* was noticed between November and January. This suggests that spawning seasons of *C. sandwicensis* may occur right after this period, whereas the lowest GSI was found from March to August, this could probably be the resting season of the species. Similarly, the same GSI pattern (**Figure 1**) of males and females *C. sandwicensis* suggested synchronized spawning of male and female *C. sandwicensis* in the wild [10].

| Date of sampling | n | Shell length (cm) | Total weight (g) | Body weight (g) | Gonad weight (g) | GSI (%) |
|---|---|---|---|---|---|---|
| November 12, 2011 | 13 | 4.26 ± 0.59 | 15.3 ± 5.49 | 5.93 ± 2.43 | 1.68 ± 0.92 | 26.8 ± 6.27 |
| December 04, 2011 | 30 | 3.46 ± 0.51 | 7.67 ± 3.63 | 2.54 ± 1.39 | 0.31 ± 0.22 | 11.7 ± 5.22 |
| January 31, 2012 | 17 | 3.55 ± 0.96 | 9.31 ± 5.74 | 2.97 ± 2.40 | 0.93 ± 1.06 | 22.9 ± 12.4 |
| February 28, 2012 | 27 | 3.11 ± 0.31 | 4.83 ± 1.74 | 1.86 ± 0.66 | 0.17 ± 0.09 | 9.05 ± 3.65 |
| March 28, 2012 | 16 | 2.86 ± 0.48 | 4.68 ± 2.62 | 1.64 ± 0.71 | 0.20 ± 0.09 | 12.0 ± 5.41 |
| April 24, 2012 | 12 | 3.26 ± 0.58 | 5.20 ± 2.47 | 1.81 ± 1.03 | 0.15 ± 0.14 | 8.73 ± 5.31 |
| May 28, 2012 | 12 | 3.27 ± 0.56 | 5.20 ± 2.47 | 2.90 ± 3.50 | 0.14 ± 0.13 | 8.10 ± 5.25 |
| June 28, 2012 | 17 | 3.17 ± 0.16 | 5.88 ± 0.91 | 2.19 ± 0.55 | 0.17 ± 0.11 | 7.25 ± 3.69 |
| July 21, 2012 | 23 | 3.25 ± 0.43 | 5.98 ± 1.67 | 2.21 ± 0.78 | 0.16 ± 0.11 | 7.19 ± 4.51 |
| August 03, 2012 | 12 | 3.95 ± 0.50 | 9.49 ± 3.91 | 3.47 ± 1.50 | 0.30 ± 0.28 | 7.56 ± 5.37 |
| September 11, 2012 | 20 | 3.43 ± 0.44 | 5.43 ± 2.19 | 1.89 ± 0.91 | 0.28 ± 0.29 | 11.5 ± 7.43 |
| October 05, 2012 | 21 | 3.71 ± 0.92 | 7.07 ± 5.38 | 2.86 ± 2.01 | 0.65 ± 0.73 | 18.7 ± 8.77 |
| November 25, 2012 | 27 | 3.96 ± 0.49 | 8.75 ± 3.21 | 3.73 ± 1.47 | 1.31 ± 0.73 | 31.8 ± 7.72 |
| December 30, 2012 | 19 | 3.96 ± 0.83 | 10.2 ± 8.64 | 4.10 ± 3.77 | 1.52 ± 1.94 | 28.4 ± 3.75 |

**Table 1.**
*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.*

## 2.2 Gonad development stage

The maturity stages of gonad development of Hawaiian limpet *C. sandwicensis* were reported and classified in **Table 2** and **Figure 2**. Multiple development stages (**Figure 2A**) were observed in the same ovary of female during the final maturation season. **Figure 2D** showed resting stages (April to August), because oocytes were not of clear formation from the ovary cell wall. Similar observation was made for gonad of the male (**Figure 2E**). The testes were densely packed with spermatozoa which appeared as dark blue stained by hematoxylin. Sperm were less densely in the male gonad (**Figure 2F**).

## 2.3 Sexual determination

Sexual determination of all limpet species is not known from external morphology. Render and maturation status of any limpet species could only be sexed after killing and dissecting. Our efforts were trying to examine ripeness of live animal

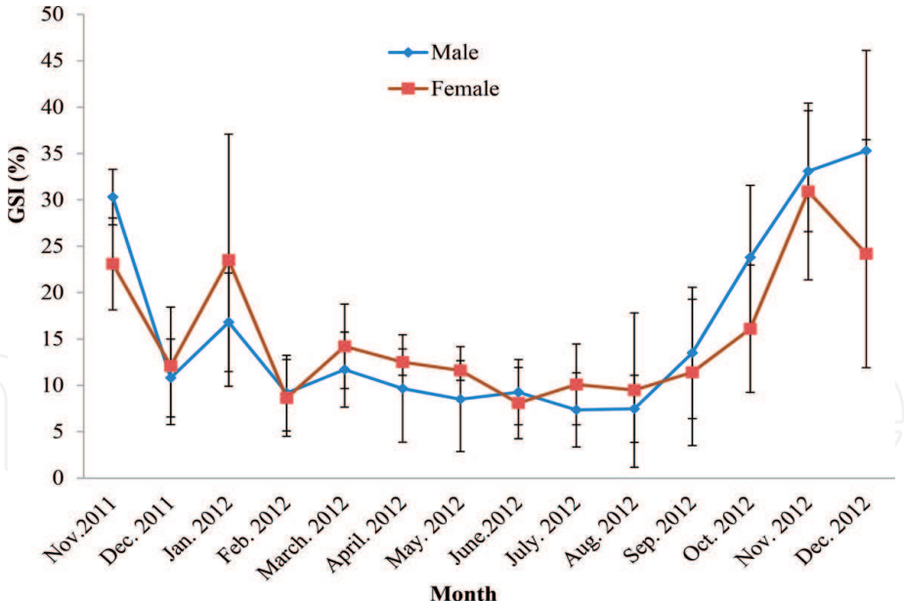


**Figure 1.**
*Seasonal changes in GSI of males and females limpet* C. sandwicensis.

| 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 μm |
| 2 | Early development | Nucleus enlarged, oocyte diameter about 7–10 μm. 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 to 100 μm |
| 4 | Ripe | Ovaries are swollen with dark brown color. Oocytes diameter ranging from 110 to 130 μm. The testis is dense with spermatozoa, milky white and/or 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. |

**Table 2.**
*Maturation stages of gonad of limpets [10–12].*

without killing them. We eventually found a way to assess the gonad, by placing them upside down on a table or putting them close to the edge of a substrate. When they try to attach to the substrate, they move their foot toward the substrate, and sometimes the gonads may be seen from the top of their head. Males were identified if the animals had milky white gonad near the edge of the shell, as shown by the arrow in **Figure 3A**. The gonad of the ripe female was dark brown or dark green in color (**Figure 3C**). It is noticed that this way, it can only be conducted when the animal reaches maturity stage or during the spawning season. Ultrasound was also an option method, but it's inconvenient and is not a practical way.

## 2.4 Fecundity and maturity size

Absolute fecundity (F) of mature female (n = 5) limpet *C. sandwicensis* is varied, and related maturity stage and body weight of animal found that total

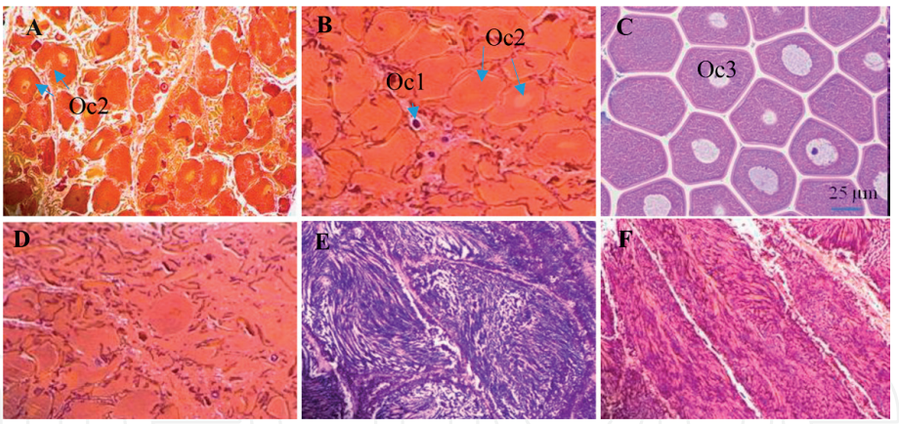


**Figure 2.**
*Cross sections showing stages of limpet* C. sandwicensis *gonad development. (A) Most oocytes in early development stages in ovary of female, (B) oocytes deforming shape in the ovary, (C) ripe stage and some oocytes were still in late development stage, (D) resting development stages of female, (E) mature male gonad with dark blue stained by hematoxylin, and (F) spermatic in gonad of male.*

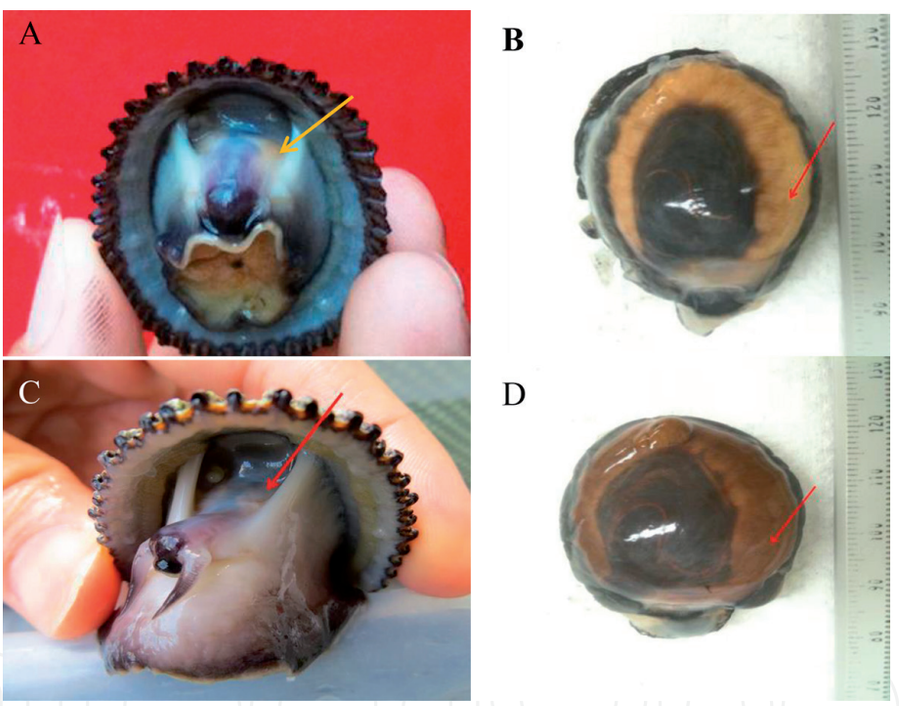


**Figure 3.**
*Determination of sexually mature male and female limpet. (A) Live limpet before dissecting, the arrow shows a sign where mature male gonad could be identified; (B) soft body tissue was removed off the shell, the mature male gonad (milky white) took up all the space around the digestive gland (dark color). This supports the location of mature gonad where it was seen as pointed in picture A; (C) live female limpet before dissecting, the arrow points where mature female's gonad would be seen; (D) showing a dark brown mature female gonad with shell removal.*

eggs counted ranged from 42.080 to 157.000 eggs per g body weight (BW). Fecundity was plotted against body weight with linearly correlated to body weight ($P$ = 0.019), and the best is described by the equations F = 28.4 BW − 77.3 ($R^2$ = 0.96). The monthly recorded GSI data combined with histology analysis showed that the Hawaiian limpet *C. sandwicensis* would attain sexual maturity size about 1.5–2.0 cm in shell length. Other studies also found that *C. sandwicensis* attained reproductive stage at shell length of 2.3–2.5 cm or larger [2].

## 3. Seed production

### 3.1 Maturation culture

Two experiments were conducted to induce final maturation of limpet *C. sandwicensis* in the laboratory conditions. The first trial is formulated feed with the addition of arachidonic acid (ARA) into diet. The experimental diet is described and shown in the previous studies [12, 13]. In brief, limpet was fed with three diets including: control diet (without additional ARA), diet 2 containing 0.20% ARA, and diet 3 (0.33% ARA). Nice adult *C. sandwicensis* (3.07 ± 0.22 cm in shell length) species were fed with these diets for 90 days. Each limpet was randomly placed into its own colander of 20 cm diameter. The colanders were placed in aquaria (150 L) with a recycled water flow rate (15 L $min^{-1}$). Seawater was exchanged weekly of about 30%. The experiment was conducted under ambient photoperiod and temperature ranging from 23 to 25°C. Salinity was maintained at 35. Prior to the beginning of the experiment, several limpets were randomly selected among the group and dissected to obtain initial GSI and gonad development status. During the experimental period, three animals were randomly examined monthly to assess maturation status as described in Section 2.3. At the end, their gonads were extracted and weighed to obtain the gonad's weight for the calculation of the GSI.

The result showed that gonad of limpet fed with diet containing ARA increased three times higher than the GSI of animals that fed with the control diet (**Table 3**). There was a significant difference ($P < 0.05$) in GSI of animal that fed with diet incorporated with ARA as compared to those fed with control diet. There was no significant difference in GSI of those limpets fed with both diets 0.2% ARA and 0.33% ARA with the same ARA/EPA ratio of 0.70.

In the following trial, the final maturation of limpet was induced by using OvaRH (Syndel Laboratories Ltd. Canada) which is a synthetic salmon GnRH analog (sGnRHa). The hormone was injected directly into the gonad of limpets. Twelve limpets (9.17 ± 3.17 g/ind.) were tagged and weighed. Each limpet received a total of five to seven injections, at 7-day intervals at dose of 250 ng/g body weight (BW). The control treatment was run without hormone injection. During the period, experimental limpets were held on biofilm aquaria with water movement by an aquarium biofilter pump (567 L per hour). The maturation of limpet was examined weekly by randomly selected and sacrificed two limpets in each treatment. Their gonads were collected for calculation of GSI, and a piece of gonad was immediately fixed in 10% formalin for histological examination. The experiment was conducted during the final maturation and spawning season.

| Day | Parameter | Control | 0.2% ARA | 0.33% 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[a] | 121 ± 9.42[a] |
| 75 | GSI (%) | 6.11 | 24.5 | 23.7 |
| | Egg size (μm) | — | 123 ± 4.23[a] | 121 ± 5.93[a] |
| 95 | GSI (%) | 4.21 ± 0.82[a] | 10.8 ± 4.47[b] | 15.5 ± 5.47[b] |

*[a,b] The same letters in the row indicate no significant difference in eggs sizes, the empty grids indicate no egg was observed.*

**Table 3.**
*Gonadal somatic index and egg size of limpet fed different dietary ARA for 95 days.*

The results showed that the gonad of limpet was rapidly increased after 3 weeks with three injections (**Figure 4**). The GSI of limpet *C. sandwicensis* increased rapidly from initial 12.0–28.3% after the third injection and reached 32.9% for the final maturation stage after the fourth injection. GSI of limpets in the control group remained the same until finishing the experiment.

It is reported that the reproduction of aquatic animal is controlling by external and internal parameter factors such as photoperiods, food availability and hormone regulation. Therefore, our study focused on three factors including photoperiod, nutrient requirement as the ratio of highly unsaturated fatty acid arachidonic acid (ARA) and eicosapentaenoic acid (EPA), and gonadotropin-releasing hormone. In our hands photoperiod is important for the maturation of limpet. 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 [11, 12]. This showed the role of environmental conditions in the regulation of the timing of the reproductive cycle of limpet. For example, the limpet *C. exarata* was found that the reproductive resting phase coincided with day length above 13 h [10], suggesting that a higher 13 h day length could inhibit gametogenesis of limpet. Photoperiod has also been reported to be influential on reproductive cycles of many marine invertebrates [14, 15].

Final maturation of *C. sandwicensis* was successfully induced by the addition of arachidonic acid (ARA) into diet to obtain an appropriate ARA per eicosapentaenoic acid (EPA) ratio. Arachidonic acid serves as a precursor for the synthetic of prostaglandins which are functional for reproductive process [16]. Prostaglandins play a critical role during the ovulatory process in teleost fishes [17]. Our previous study found that *C. sandwicensis* preferred to feed on benthic diatoms in the wild [18]. Several benthic diatoms such as *Nitzschia*, *Amphora*, and *Navicula* were predominant in the stomach content of *C. sandwicensis*, and literature studies found that these diatoms contained high level of ARA and EPA [19–21]. Our review found that an ARA/EPA ratio of about 0.70 was found in several benthic diatoms such as *Nitzschia* and *Chaetoceros* suggesting that this would be a good starting point for experimental diet on adult limpet *C.*

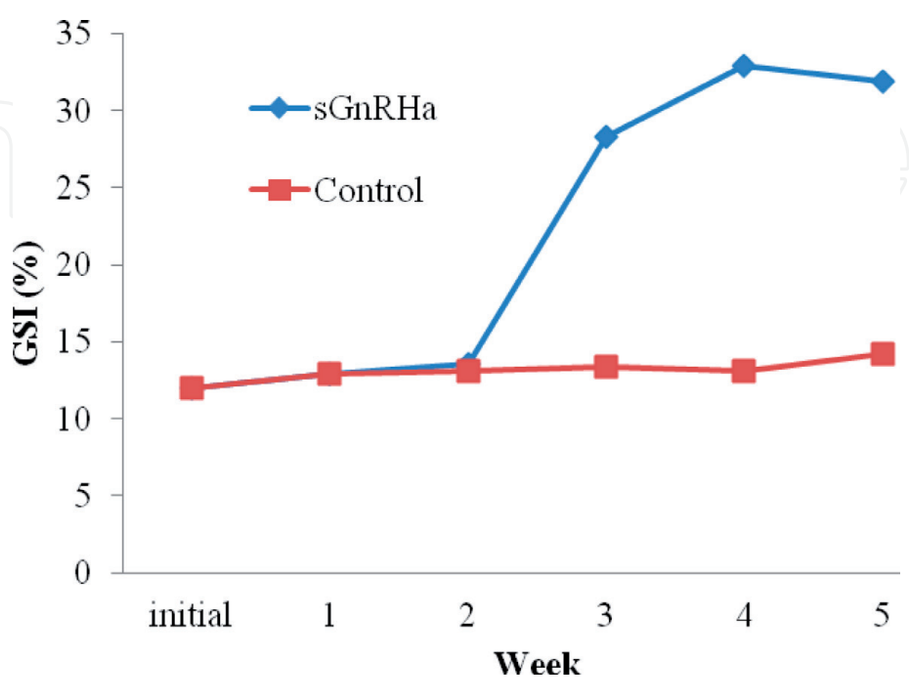


**Figure 4.**
*Gonadosomatic growth of limpet* C. sandwicensis *by hormone induction.*

*sandwicensis*. Thus, experimental trial on different ARA to EPA ratios of 0.70 was conducted; as a result, *C. sandwicensis* reached final maturation [12]. This result provided significant data on the effect of ARA/EPA on maturation of limpet and gastropods as well.

GnRH-like peptides that existed in the central nervous system and peripheral chemosensory organ of sea hare *Aplysia* were detectable by antisera against mGnRH [22]. 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 abalone [23, 24], and the existence of GnRH-like peptide in the neural ganglia was determined by using immunohistochemistry and reverse-phase high-performance liquid chromatography [24, 25].

The Hawaiian limpet *C. sandwicensis* were also induced to final maturation using salmon GnRH analog (sGnRHa) at dose of 250 ng/g BW. The sGnRHa stimulated gonad development and final maturation in limpet in 5 weeks when they injected at 7-day intervals at low concentration 250 ng/g BW [13]. The GSI increased significantly from the third week of injection and developed rapidly and reached to the maximum level after 4 weeks of injection as compared to the control, which did not show gonadal development (**Figure 4**). This shows that GnRH also involved in regulating reproductive development in limpet. Similar finding was also reported in abalone; the adult abalone was induced to 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 1000 ng/g BW [23]. The existence of GnRH-like peptides in the neural ganglia and ovary of the abalone [23, 24] and the existence of GnRH-like peptide in the neural ganglia were determined by using immunohistochemistry and reverse-phase high-performance liquid chromatography [24, 25]. GnRH-like peptides that existed in the central nervous system and peripheral chemosensory organ of sea hare *Aplysia* were detectable by antisera against mGnRH [22]. These GnRH-like peptides controlled egg laying of *Aplysia*. The mammalian GnRH analog was known to stimulate maturation and induced spawning in abalone [23]. The responses of molluskan to environmental cues are controlled by hormones, and the principal sources of hormones within molluscan nervous system are neurosecretory cells [26]. Our results suggest that diatom blooms may be the environment cues. GnRH could stimulate reproductive process by acting directly on the gonad in limpet. Both limpet and abalone are marine gastropod species. This process would be also facilitated by the reproductive photoperiod, and/or the right photoperiod would stimulate the increased secretion of luteinizing hormone and follicle-stimulating hormone that enhances the reproductive process in limpet *C. sandwicensis*.

### 3.2 Spawning induction

Two different spawning methods were conducted to examine the optimal method of spawning for the Hawaiian limpet. The first method was conducted using hydrogen peroxide. Hydrogen peroxide is a traditional method used for spawning induction in abalone. **Figure 5** shows the addition of $H_2O_2$ to seawater is believed to produce hydroperoxy free radicals ($HOO^-$) and peroxy radicals ($OO^-$); these radicals of activated oxygen suitable for the cyclooxygenase catalyzed addition of prostaglandin [27–29].

Experimental animal. Limpet broodstock (>3.0 cm in shell length) were collected at the shoreline from a remote area on Oahu island. They were held on biofilm aquaria for 2 days before use for the experiment. Sexually matured broodstock were selected as described in Section 2.3. Eight matured limpets

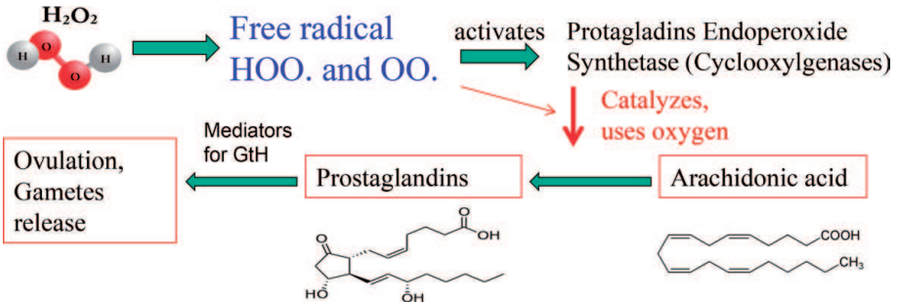


**Figure 5.**
*Mechanism of hydrogen peroxide in spawning induction of mollusk species.*

(approximate sex ratio, male:female = 1:1) were selected from the holding tank then placed into a spawning container (3 L) with fresh clean seawater for each trial. The spawning container was gently aerated, and pH in the spawning container was first adjusted to pH 9–9.5 by 1 M of Tris-base for about an hour. Thereafter, stock 6% of $H_2O_2$ was slowly added to spawning container to obtain the desired concentration. The broodstock were exposed to five different concentrations of $H_2O_2$. These are control (without $H_2O_2$), $0.6 \times 10^{-2}$%, 1.20%, $1.49 \times 10^{-2}$%, and $1.80 \times 10^{-2}$%. The exposing time ranged from 5 to 45 min depending on the response of animals. Then the spawning activities were observed. The results show that the highest number of spawners was induced at $0.6 \times 10^{-2}$% and no mortality occurred in the 24 h after spawning. Most of animals died at $1.49 \times 10^{-2}$% and $1.80 \times 10^{-2}$% in the 24 h after exposure to these levels. These results highlighted the nonspecific toxic effect of the chemical. Similarly, at this level all animals were dead eventually, but this level induced 10–15% spawning [10]. However, we concluded that this method may not be used as a practical method and not recommended for spawning induction in limpet. This was due to a nonspecific effect, and the broodstock eventually died within a week after being exposed to $H_2O_2$. The second method with GnRH at dose of 1000 ng/g BW may be considered as the most practical induction spawning method for limpet because there were no mortalities occurring after spawning.

This could probably be due to the instability of $H_2O_2$. The $H_2O_2$ was fresher, and we ordered before use. No mortality occurred in the 24 h 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 $H_2O_2$. We used this level in further spawning the trials. The limpet 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 limpet by GnRH is effective only on ripe *C. sandwicensis*.

### 3.3 Embryonic and larval development

Different larval development of *C. sandwicensis* is shown in **Table 4**. There were 18 distinct stages of larval development of *C. sandwicensis* in this study. Spawned eggs were 111 ± 5.64 μm (**Figure 6a**). The first polar body appeared in about 30–45 min after spawning indicating fertilized eggs (**Figure 6b**). The two-cell

| 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 girdle 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 |

**Table 4.**
*Embryonic and larval development of limpet* C. sandwicensis *at (22°C).*

stage (stage 3) was found within 2 h after spawning (**Figure 6b**). About 10 h post-fertilization, protrochophore stage with cilia appeared (**Figure 6b**). Larvae started hatching out at 12–14 h. The length and width of free swimming larvae were 85.5 ± 9.5 μm and 79.6 ± 7.9 μm, respectively. Larvae continued to develop velum from cilia, and apical region became flat for shell formation in about 18–20 h after spawning (**Figure 6o** and **p**).

### 3.4 Larval rearing

Several studies [10, 11] on settlement of *C. sandwicensis* larvae on different combinations of diatom and pelagic algae were conducted. The results showed that mixture of diatom *Amphora* and pelagic *Palova* induced the highest survival rate (21.7 ± 7.07%) of settled larvae. Diatom *Nitzschia* seemed not to be preferred by *C. sandwicensis* larvae because the observation noticed that high mortalities occurred from 4 to 6 days. Pelagic algal *Palova* may be preferred over *Isochrysis*. Among the surviving larvae, all of them settled after 3 days and fed on diatoms. On the other hand, different plate substrates reported to be affected on larval settlement of gastropod species, such as abalone of roughened plexiglass, and corrugated plastic sheet, and the rubberized canvas seemed to be preferred for settlement over fibrocement board. The results of our study were higher than previous study which was attempting to induce the settlement of Hawaiian limpet *C. sandwicensis* larvae on different substrata [10]. She found that mylar plastic and plexiglass induced a significantly higher larval settlement compared to glass, smooth and rough basalt

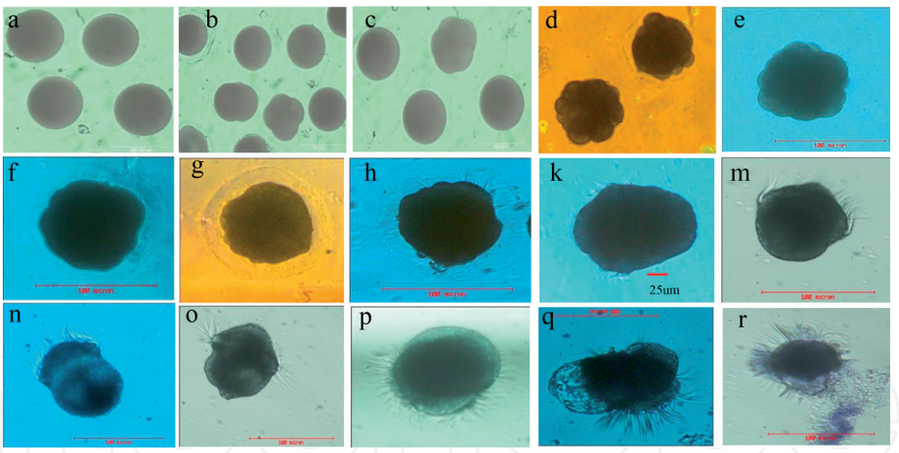


**Figure 6.**
*Embryonic development stage of Hawaiian limpet* C. sandwicensis. *(a) Spawned egg and stage 1 spermatozoids; (b) stages 2 and 3, discharge of polar body and first cleavage (2 cells), and stage 4, second cleavage (4 cells); (c) stage 5, third cleavage (8 cells); (d) stages 6–7 morula and blastula; (e) stage 8, gastrula; (f and g) stage 9, appearance of cilia forming the prototrochal; (h) stage 10, trochophore larvae ready for hatch out; (k) stage 11, trochophore larvae free swimming; (m) stage 12, trochophore free swimming larvae with extend cilia; (n) stage 13, complete girdle, cilia develop and apical; (o) stage 14, early larvae shell formation; (p) stages 15 and 16, veliger larvae exhibiting flat apical from larval shell and complete developed velum and cilia; (q) stage 17, appearance of eye spot; (r) stage 18, appearance of muscle attached.*

rocks, coral skeleton, and textured and untextured plastic. However, the settlement rates were very low ranging from 1.58 to 7.73%. It could probably be due to inappropriate benthic diatoms.

The role of benthic diatoms *Navicula*, *Amphora*, *Nitzschia*, and others were reported to best diatom species induced the settlement and metamorphosis of abalone larvae [19, 30, 31]. The effects of different benthic diatoms grown on different plate substrates on metamorphosis of the tropical abalone *Haliotis asinina* were reported by [31]. They found that mixture of diatoms induced significantly higher metamorphosis rate of abalone larvae than other group including *Amphora*, *Amphora* + *Nitzschia*, and *Nitzschia* with any plate substrate. This suggested that mixture of benthic diatoms is better than single once. Another study also found that a mixture of benthic diatoms consisting of *Navicula* and *Amphora* produced a significantly higher growth and survival rates for abalone larvae *H. discus hannai* than monocultures benthic diatoms [19]. The report showed that the monocultures of benthic diatoms produced a poor growth and did not support survival for more than 2 weeks especially *Nitzschia*. The authors also stated this could be due to the 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* [19]. These results support our study that mixture of diatom and pelagic algae induced better survival rate of Hawaiian limpet and mixture of diatom *Amphora* and pelagic *Palova* would be recommended for future use of larval rearing of the *C. sandwicensis*.

## 4. Culture system

There is a lack of study on aquaculture system for limpet species as well as the Hawaiian limpet *C. sandwicensis*. We have attempted to raise the limpet *C. sandwicensis* in system with water flow through, but transfer mortality is a challenge because the animals cling tightly to the cultured tank walls. It was hard to get them off the wall without injury. Later we found that putting plastic sheets as tank liners solves this problem (**Figure** 7).

**Figure 7.**
*A circular holding biofilm tank without plastic liner, and three aquaria with plastic sheer liner above, used for the second and following holdings.*

Rocky habitat and adhering to the substrates are problems. Limpet *C. sandwicensis* attach to the washing rocks in the wild. They cling to the culture tank with their muscular foot. It indicates that physical damaged may happen while removing them off the tank's wall. Similar observation has 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 hemolymph [32]. Eventually, we developed plastic tank liners that were our breakthrough for transferring animals from one tank to another. Our study was the first to reveal that the Hawaiian limpet *C. sandwicensis* was healthy and fed well in the experimental aquaria without intermittent water sprayed or dump tanks.

## 5. Feed development and nutrient requirement

### 5.1 Development of formulated feed

We [18] began our studies in this area with several preliminary tests on biofilm because *C. sandwicensis* ate biofilm well which should be close to their natural diet. We also tested several dry diets, gelatin, and agar diets. We discovered that several were preferred and some were not. Several chemical attractants were tested including betaine, gamma aminobutyric acid (GABA), and dimethyl propiothetin (DMPT), but these did not enhance feeding. Among the feeds tested in a preliminary way, fish meal and soybean meal as well as feeds incorporating biofilm were preferred. Eventually, we found that *Porphyra* preparations could replace biofilm as a feeding stimulant in formulated feed.

Nutrient requirement was our next step to develop the commercial feed available for limpet, and the authors assumed that the nutrient requirement of limpet and abalone is the same as they are marine gastropod [18]. For abalone, a series number of researches had been done, and the optimum nutrient requirement as protein, carbohydrate, and lipid was focused. However, the results still varied among researchers. For example, the protein requirements of abalone found by previous studies [33, 34] were higher than those reported in the previous studies [35–37]. Poor growth was found for abalone when the animal was fed with formulated diet containing amino acid profile that does not match the animals' tissue [38]. Moreover, other studies [39, 40] found that a significant lower growth rates when abalone fed with dried kelp *Ecklonia maxima* and *Laminaria*. Therefore, these studies raised the hypothesis that the growth rate of abalone is related to the degree of the amino acid profile of feed and the amino acid profile of tissue.

## 5.2 Protein and carbohydrate requirement

Based on the results of previous study [18], further studied on the determination of protein and carbohydrate requirement for Hawaiian limpet *C. sandwicensis* [41].

Experimental animals. Adult *C. sandwicensis* limpets (shell length above 3.0 cm) were collected from a remote area in Oahu, Hawaii, used for this study. After collection they were immediately placed into a 14 L ice plastic insulation box with plastic liner and then transported to the laboratory at the University of Hawaii in Monoa. The limpet was held in a plastic aquaria 150 L with water flow for a week; during this period, the animal were fed with the experimental diet and the commercial algae *Porphyra tenera* or *yezoensis*, known as Nori (Nishimoto Trading Co. Ltd., Korea).

Experimental diets. Formulations of dietary protein and carbohydrate levels are shown in **Table 5**. The first trial was done for dietary protein level, following by dietary carbohydrate. For carbohydrate trial, four different dietary carbohydrate levels of 18, 27, 32, and 37% were tested. The amino acid profiles of *C. sandwicensis* tissue and of the dietary protein in trial 1 were analyzed at the Aquatic Feed and Nutrition Laboratory, Oceanic Institute, Hawaii, USA, according to the described method [42]. The results are presented as A/E ratio (**Table 6**). Most of the essential amino acids of diets were identical and/or close to the amino acid profile of *C.*

| **Ingredient** | **Dietary protein trial 1** | | | | | **Dietary protein trial 2** | | | | **Carbohydrate trial** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **270** | **320** | **370** | **420** | **470** | **210** | **300** | **350** | **500** | **180** | **270** | **320** | **370** |
| Fishmeal | 16.5 | 19.5 | 22.5 | 25.5 | 28.5 | 13.4 | 17.0 | 21.0 | 30.4 | 16.5 | 16.5 | 16.5 | 16.5 |
| Defatted soybean | 11.5 | 14.5 | 17.5 | 20.5 | 23.5 | 11.0 | 12.7 | 16.6 | 24.4 | 11.5 | 11.5 | 11.5 | 11.5 |
| Krill meal | 4.5 | 7.5 | 10.5 | 13.5 | 16.5 | 7.1 | 8.0 | 11.0 | 16.1 | 4.5 | 4.5 | 4.5 | 4.5 |
| Porphyra[1] | 14.0 | 14.0 | 14.0 | 14.0 | 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 | 5.3 | 4.3 | 0.8 | 15.4 | 26.9 | 33.7 | 40.5 |
| Diatomaceous earth | 29.2 | 21.9 | 14.6 | 73.0 | 0.0 | 36.8 | 35.2 | 25.8 | 7.3 | 30.9 | 19.4 | 12.6 | 5.8 |
| Alginate | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Corn/fish oil[2] | 2.5 | 1.9 | 1.2 | 0.6 | 0.0 | 2.32 | 1.4 | 0.9 | 0.6 | 1.0 | 1.0 | 1.0 | 1.0 |
| Vit. mix[3] | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Cholesterol | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.2 | 0.2 | 0.2 | 0.2 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Water | 100 | 100 | 100 | 100 | 100 | 133 | 133 | 133 | 133 | 100 | 100 | 100 | 100 |
| Analyzed and calculated nutrient "as fed" | | | | | | | | | | | | | |
| Crude protein | 26.5 | 31.7 | 37.0 | 42.4 | 47.7 | 21.2 | 30.5 | 35.8 | 49.2 | 26.5 | 26.5 | 26.5 | 26.5 |
| Crude lipid | 4.97 | 4.97 | 4.97 | 4.97 | 4.97 | 5.13 | 5.13 | 5.13 | 5.13 | 3.47 | 3.47 | 3.47 | 3.4.7 |
| Carbohydrate | 17.5 | 17.8 | 18.1 | 18.3 | 18.0 | 11.0 | 11.0 | 11.2 | 11.4 | 18.0 | 27.0 | 32.0 | 37.0 |

[1]*This is commercial seasoned seaweed known as nori or the red algae Porphyra tenera or yezoensis. Nishimoto Trading Co. Ltd., Korea.*
[2]*Corn oil and menhaden oil (1:1; v/v).*
[3]*Commercial vitamin mix (NRC 1981) was kindly provided from Dr. Warren Dominy (Oceanic Institute).*

**Table 5.**
*Composition of formulated diet (% dry matter).*

| Essential AA | Preliminary protein trial (trial 1) | | | | | | Second protein trial | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Tissue | 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 |

**Table 6.**
*The A/E ratio [(each EAA/total EAA) × 1000] amino acids of dietary protein and animal tissue.*

*sandwicensis* tissue except for Arg and Thr which were lower in the experimental diets compared to the tissue.

The process of feed preparation for extrusion of all diets was based on the methods described by the previous study [18, 41]. In brief, fish meal, soybean meal, and krill meal were mixed thoroughly with other ingredients. Wheat flour was used as starch, and diatomaceous earth was used as filter to balance in the diets. Wheat flour and alginate were gelatinized in boiling water (about 25% of total dried weight basis) before being mixed with other ingredients. Other ingredients were then mixed thoroughly with the gelatinized solution; thereafter the mixed (paste) was heated in boiled water bath again for about 2 min. The paste was shaped into sheets about 1.0 mm thickness and then cut into 1.2 $cm^2$/pieces and dried naturally in laboratory conditions for about 1–2 h. The pieces were then sealed in a plastic sample bag and stored at −4°C until use.

Each limpet was randomly placed into its own colander of 20 cm diameter (**Figure 8**). The colanders were placed in aquaria (150 L) with a recycled water flow rate (15 L $min^{-1}$). Nice limpets were used for diet, and the experiment was run for 90 days.

The growth of animals in weight (g) and shell length (cm) was measured monthly. The growth was expressed in terms of specific growth rate (SGR), weight gain, and shell length increasing. The shell length was measured with an electronic digital caliper (0.01 μm), and the weight was determined with an electronic scale (0.01 g error) for every 4 weeks:

SGR = {(lnWf − lnWi)/T} × 100, where Wf is the final weight, Wi is the initial weight, and T is the total day of the experiment.

The result showed that the growth response of *C. sandwicensis* in terms of weight gain (%) of animals in dietary protein trial 2 was fitted into quadratic models (**Figure 9**). The best fit for the estimation of optimal protein level could be described as $Y = -0.0003x^2 + 0.234x - 21.8$ ($R^2 = 0.96$). The trend of growth showed that maximum weight gain appeared to be about 35% dietary protein.

The response of *C. sandwicensis* in weight gain to dietary carbohydrate levels was then fitted to quadratic models (**Figure 10**). It shows that the weight gains of *C. sandwicensis* progressively increased and reached their maximum value at a carbohydrate level of about 27%, which could probably be described as $Y = -0.0012x^2 + 0.64x - 56.7$ with the correlation value of $R^2 = 0.91$.

**Figure 8.**
*Experimental colander with an* C. sandwicensis *on it; a small square is a piece of feed.*

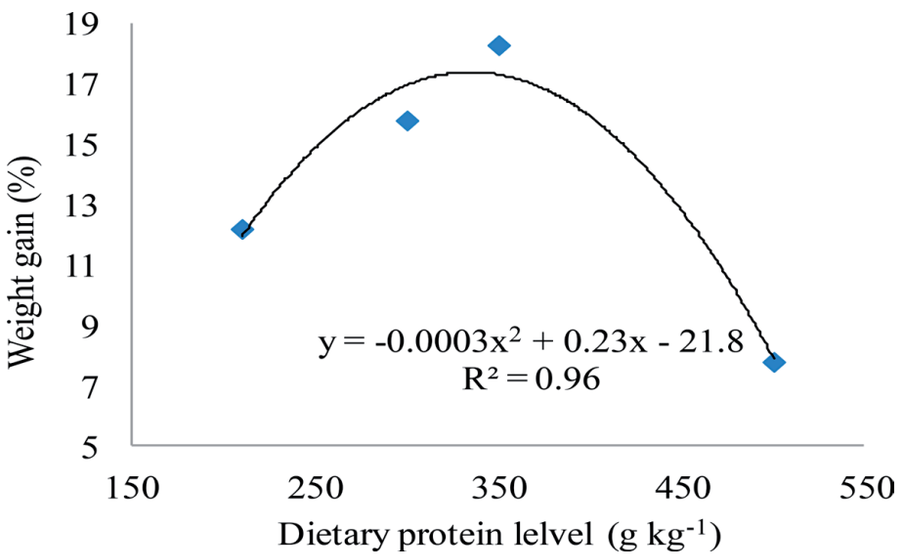


**Figure 9.**
*Relationship between weight gain and dietary protein level of trial 2 for* C. sandwicensis *for 60 days.*

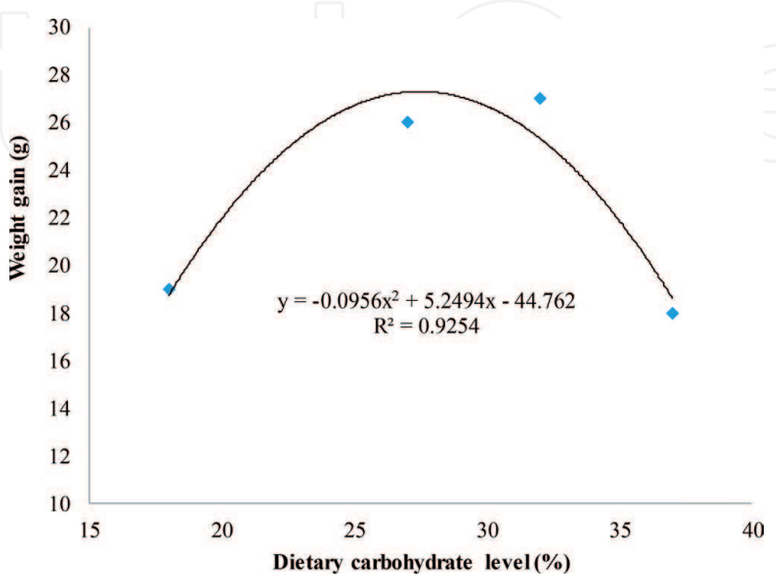


**Figure 10.**
*Relationship between weight gain and dietary carbohydrate level for* C. sandwicensis.

### 5.3 Energy requirement

Recent study found that limpet *C. sandwicensis* required no specific effect on dietary protein to energy (PE) ratio when the animal was offered with diet containing various PE ratios ranging from 87.2 to 102.9 mg/kcal [43]. There was no significant effect on growth performance of limpet among the diets, but a PE ratio of 87.2 mg/kcal produced the best tissue growth.

## 6. Conclusion

This chapter provides scientific basis for the development of aquaculture techniques for the Hawaiian limpet *C. sandwicensis*. Several reproductive characteristics of the Hawaiian limpet *C. sandwicensis* were investigated such as natural spawning season (November to January), maturity size (above 1.5 cm shell length), and gonad development stage (5 stages), examining sexually matured of male and female animals. The second important issue is seed production. Induction of final maturation using dietary ARA/EPA ratio of 0.70 and injection of sGnRH at dose of 250 ng/g BW is recommended. Induction of spawning by using sGnRHa is an applicable technique and was the most practical method with no mortality occurring. Pelagic algal *Palova* and benthic diatom *Amphora* induced good survival rate for larval settlement and are potential algal species for commercial hatchery of larval rearing for *C. sandwicensis*. An effective method of using plastic liner and/or colander for handling and potential use for culture system of limpet was also developed. A practical commercial feed with good palatability, producing good growth performance at 35% dietary protein, 32% for carbohydrate and protein to energy (PE) ratio ranging from 87.2 to 102.9 mg/kcal could be used for commercial limpet feed.

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