8. DISCUSSION

8.1 <u>Economical significance of *Bellamya bengalensis* on the basis of potential nutrient requirement than other economically important animal sources</u>

This is true that socio-economic conditions limit the capacity to produce and purchase of any food items. It was thought that if people have access to a sufficient quantity and variety of foods, they should meet their nutritional needs. The current practice of evaluating nutritive significance of *B. bengalensis* included the abundance of the micronutrient density, that reflected its flesh value, in terms of much lower cost than the other commodities like fish, meat etc. It is true that, if any product does not carry any inherent qualitative importance, that product cannot able to sustain within any marketing world. From the immemorial time being it was observed that *B. bengalensis* act as a healthy edible product to different economical class of people. And also for those people who cannot able to purchase fish or meat in their daily life style. Not only that to cure certain diseases they depend on *B. bengalensis*. In our present study it was seen that *B. bengalensis* was carrying appreciable amount of required nutrient ingredients i.e. protein, carbohydrate and lipid. Each of them plays vital role to maintain our physiological activities.

8.2 <u>Proximate composition of protein and amino acid in the flesh of *Bellamya* <u>bengalensis</u> as well as its role to regulate human physiological activities</u>

Protein is the most important constituents in our food materials which catabolise as a source of energy, takes part in the formation of hormones, enzymes and a wide variety of other biologically important substances such as antibodies, hemoglobin etc. Protein plays vital role for the growth and development during childhood, adolescence, and pregnancy. So in this regards, we are very protein conscious and intake protein reach food materials. In our present study it was observed that crude protein content in *B. bengalensis* was 48.65 \pm 0.85%. Whereas protein content in other edible Molluscs were 8.27 %, 12.36 %, 6.46 %, 12.92 % and 12.87 % in *Pila globosa, Melania tuberculata Lamellidens marginalis, Anisus convexiusculus* (Baby *et.al.*,2010) and *Helix aspersa* (Cagiltay *et.al.*, 2011) respectively. On the other hand it was observed that the avg. protein content in several fishes like; Carp 42-43 %, (Dabrowski, 1977). While estimated protein content was reported among red meat group, chicken 30% (Dolson, 2014), Pork 22% (Dolson,2014), Beef 28% (Dolson,2014) etc. Therefore, in this regards it was cleared, this significant amount of protein which were not less than (rather, greater than) the fish and red meat communities. More than that, from the monetary point of view, it held much cheaper market rate than fish and red meat groups (Chakraborty, Mukherjee & Maity, 2014).

It was narrated by (WHO *et.al.*, 2007) that mean protein requirement for older, adults and the elderly lied in between 0.66 to 0.75 gm / kg body wt. /day. After making comparison with the (WHO's 2007) report, it was assumed, *B. bengalensis* confirmed such amount of protein and amino acid, which were essential for older, adults and the elderly to maintain their daily physiologic process.

Though for human, essential amino acids are Phenylalanine, Valine, Threonine, Tryptophan, Isoleucine, Methionine, Ieucine, Lysine, and Histidine (Young,1994) and along with Cysteine, Argenine, Tyrosine are required for infants and growing children (WHO *et.al.*,2007). Whereas Alanine, Serine, Glycine, Glutamine acid etc are the non essential amino acid. In addition to that Arginine, Glycine, Glutamine, Serin etc are considered as "Conditionally" essential amino acid (Reeds, 2000 and Fürst *et.al*, 2004). However, in this experiment a lots of important amino acids were detected (Table 13),

these amino acids play vital contribution in controlling human physiological process. Their presence in this experiment (in percentage) and importance is discussed as follows: Argenine $(5.61 \pm 0.59 \%$ as per WHO *et.al.*, 2007 our daily requirement 206 mg/kg/day) is important for production of T-cells, good for people suffering from arthritis. It also helps in releasing the insulin from pancreas (WHO *et.al.*, 2007). Histidine (6.19 \pm 0.50 % as per WHO et.al.,2007 our daily requirement 0.010 gm/ kg body wt./dy) is associated with pain control, nerval deafness, production of white blood cells, aiding early digestion by stimulating stomach acid secretion treating (WHO et.al., 2007). Isoleucine (2.70 ± 0.33 % as per WHO et.al., 2007 our daily requirement 0.020 gm/kg body wt./dy) is important for the formation of hemoglobin, stabilization and its regularization of blood sugar, repairing of muscle tissues, it works with Valine (WHO et.al., 2007). Leucine (7.51 ± 0.26 % as per WHO et.al., 2007 our daily requirement 0.039 gm/ kg body wt./dy) plays its role to releases a lots of energy during any form of exercise, tissue repair after surgery, blood sugar (diabetics) control, it also works with Valine (WHO et.al., 2007). Lysine $(16.13 \pm 0.51\%$ as per WHO *et.al.*, 2007 our daily requirement 0.030 gm/ kg body wt./dy) improves concentration, preventing fertility problem, also helps in calcium absorption (WHO *et.al.*, 2007). Methionine $(0.61 \pm 0.39 \%$ as per WHO *et.al.*, 2007 our daily requirement 0.010 gm/ kg body wt./dy) assists gall bladder functions, balances the urinary tract pH, supporting the liver function, beneficial for those who are suffering from osteoporosis, and gives rise to Taurine (an important neuron-regulator in the brain) (WHO et.al., 2007). Phenylalanine $(2.10 \pm 0.36 \%$ as per WHO et.al., 2007 our daily requirement Tyrosine+ phenylalanine 0.025 gm/ kg body wt./dy) related to appetite control, blood pressure in hypotension, works with minerals in skin and hair pigmentation, produces adrenalin and noradrenalin. It is used for rheumatoid arthritis,

migraine pain (WHO *et.al.*,2007). **Therionine** (18.89± 0.88 % as per WHO *et.al.*,2007 our daily requirement 0.015 gm/ kg body wt./dy) enhance the immunity system, important role in collagen production, maintain a proper protein balance in the body, does not allow fats to build up on the liver, mood elevation or depression and skin pigmentation, manufactures adrenalin, and precursors thyroid hormone (WHO et.al., 2007). Valine (3.66 ± 0.12 % WHO et.al., 2007 our daily requirement 0.023 gm/ kg body wt./dy) regulates the metabolism, useful for the people who have just come out of drug addiction as they help to correct the amino acids deficiencies caused due to drugs, also acts as a stimulant and provide energy during times of fasting or between meals (WHO et.al., 2007). Tyrosine $(1.90 \pm 0.60 \%$ as per WHO *et.al.*, 2007 our daily requirement Tyrosine+ phenylalanine 0.025 %) is needed especially for infants regarding proper growth and development, also require for pituitary gland and the thyroid gland and it defends the skin against radiation (WHO *et.al.*, 2007). Cystiene $(0.84 \pm 0.82 \%$ as per WHO *et.al.*, 2007 our daily requirement 0.004 gm/ kg body wt./dy) is needed by infants and children, synthesized from Methionine, functional component of proteins and enzymes (WHO et.al., 2007). Serine $(7.62 \pm 0.45 \%)$ is most important non essential amino acid which is useful for brain and central nervous system, memory power, brain development, releasing hormone etc (Mothet et al., 2000).

8.3 <u>Proximate composition of fat and fatty acid in the flesh of *Bellamya bengalensis* as well as its role to regulate human physiological activities</u>

Based on Ackman (1994) classification the crude lipid content in *B. bengalensis* was located between 4-8%, which indicated presence of medium lipid level in the flesh of *B. bengalensis* (Table 12). Gass chromatograph illustrated about the presence of fatty acid content in *B. bengalensis* at significant level (P<0.05), such as palmitic, myristic, stearic, lauric, oleic, erucic, linoleic, α -linolenic, eicosapentaenoic and docosahexaenoic (Table 14). More over the isolated n-3 fatty acid group were indicating about the flesh values of *B. bengalensis*. Generally, common sources of PUFA was (mainly n–3 fatty acids) in fish, gastropods, algae etc (Babu *et.al.*,2011). It was described that the percentage of total PUFA in marine gastropods *viz. Tonna dolium* and *Phalium glaucum* were 3.55% and 3.16% respectively (Babu *et.al.*,2011). While the percentage of total PUFA in other prosobranch gastropod, *Patella depressa* was ranged between 6.04±1.99% to 10.40±1.01% (Morais *et.al.*,2003). So here *B. bengalensis* can act as a good source of EFAs in terms of PUFAs.

Some evidence suggests that people with certain circulatory problems, such as varicose veins, may benefit from the consumption of EPA and DHA, which may stimulate blood circulation, increase the breakdown of fibrin (a compound involved in clot and scar formation) (Heinze,2012). Evidently, these n–3 fatty acids can reduce blood triglyceride levels (Heinze,2012, Wak,2012 and Chua,2013) sticking to artery walls for blocked blood vessels. This also help to prevent hardening of the arteries, decrease risk of sudden death and abnormal heart rates, help for lowering of blood pressure, and prevent heart attacks. The EFAs can reduce inflammation in heart disease, inflammatory bowel disease, and rheumatoid arthritis. Regular intake of n-3 fatty acids may reduce the

risk of secondary and primary heart attack (Kotwal,2012). Not only that n-3 group of fatty acid as DHA, plays crucial role for brain and eye development of growing fetus during the pregnancy period, for maintaining and promoting health throughout the life. Thus, the Canadian Government recognized the importance of DHA n-3 (Delgado-Lista,2012). Whereas n-6 fatty acids associated with replacing saturated and trans fat with decreasing risk of heart disease, improve insulin resistance and reduce the incidence of diabetes, and also help in lower blood pressure and cholesterol levels etc (Pharmacy and Therapeutics,2008). It can be added that present research work suggested about the flesh value of *B. bengalensis* and its potential role for providing EFAs (EPA and DHA), in a satisfactory amount. This study again confirmed about the potential importance of *B.bengalensis*, starting from growing fetus to children, adults and elderly for development and maintenance of their vital physiological functions.

The ratio between n-3 and n-6 (especially alpha-linolenic vs linoleic) is a very useful index for human health on coronary heart disease, autoimmune diseases etc. However, two studies published in 2005 and 2007, found that n-3 fatty acids play extremely beneficial role in preventing heart disease in humans, while levels of n-6 fatty acids (within the ratio) was insignificant (Mozaffarian *et.al.*,2005 and Willett,2007). More over the n-3 and n-6 ratio can significantly influence eicosanoids, which regulate metabolic functions. This eicosanoids (leukotrienes, thromboxanes and prostaglandins) which, are a family of powerful, hormone-like compounds, produced in the body from the synthesis of EFA and ending with metabolism by enzymes. These eicosanoids have important beneficial biological role (immune response, relevant to arthritis, lupus, asthma, recovery from infections etc). But their excess production may create ill effect

(Liza and Castle,2010). However, researchers found that certain n-3 fatty acids are responsible for conversion into eicosanoids. Whereas, the production rates of eicosanoids were observed, faster from n-6 than n-3 fatty acids. In that case, if both n-3 and n-6 fatty acids are present in almost same ratio, they will "compete" to be transformed (Liza and Castle,2010).

Not only that, some eicosanoids, which derived from n-6 fatty acid (mainly arachidonic acid), are potentially harmful if excessive amounts build up in the body. So the ratio of long-chain n-3 and n-6 fatty acids directly affects the type of eicosanoids that are produced. These observations led to greater interest in finding ways to control the synthesis of n-6 eicosanoids. It was also observed that humans can convert short-chain n-3 fatty acids to long-chain form (α -linolenic acid to EPA and further to DHA) (Liza2010). The n-3 conversion efficiency is greater in women than in men. These conversions occur competitively with n-6 fatty acids, which are essential closely related chemical analogues which are derived from linoleic acid. Thus, the synthesis of the longer n-3 fatty acids from linolenic acid within the body is competitively slowed by the n-6 analogues. That is why, accumulation of long-chain n-3 fatty acids in tissues is more effective, when they are obtained directly from food or when competing amounts of omega-6 analogues do not greatly exceed the amounts of omega-3. Nevertheless, this appropriate ratio was reported to be present during the prenatal period for the formation of synapses and cell membranes. These processes are also essential in postnatal human development for injury response of the central nervous system and retinal stimulation (Goyens, 2006) Thus, it can be suggested that, it is a simplest way to consuming more n-3 and fewer n-6 fatty acids. In this experiment, the n-3 and n-6 ratio was observed 4.33 (Table 14). Which indicated *B. bengalensis* act as a moderately balance source of fatty acids (mainly EFA). Where the estimated value of n-3 is significantly more than the value of n-6. It should however be noted that regular consumption of *B. bengalensis*, can contribute to increase the amount of EFA in the diet. Nevertheless, more experiment in this regards can be done.

8.4 <u>Proximate composition of vitamin in the flesh of *Bellamya bengalensis* as well as its role to regulate human physiological activities</u>

Beside the protein, lipid, carbohydrate and along with their essential parts, vitamins can play an essential role to get healthy and protective body. Vitamins contain no useful energy, but act as a catalysts for reactions within the body, they serve as essential links and regulators in metabolic reactions that release energy from food. Vitamins are required in a small amount but their reflection of works is wide, such as growth and maintenance of health, metabolism, involve in control the processes of tissue synthesis, aid in protecting the integrity of the cells' plasma membrane etc. The work process of vitamins can be explain in this way that- to refresh memory, catalyst can play there, an essential role which allows a chemical reaction, using less energy and less time, under normal conditions. If this catalysts become missing, it will be attributed as vitamin deficiency. Vitamins generally do normal physical functions, in absence of which normal working capacity can be hampered. That is why people suffer from disease susceptibility (Bender, 1992). Vitamins are classified into two main groups, water-soluble and fatsoluble. Vitamin A, D etc are the fat-soluble, where as Vitamin B complex, C etc are soluble in water. Each of the stated vitamins plays a significant role for maintaining normal physiological activity.

As it was studied that *B. bengalensis* played a great role in improving visual cycle, strengthening of bones, helped in digestion and also played vital role in preventing several diseases such as- rheumatism, cardiac diseases, controlling blood pressure, rickets, calcium metabolism, nervousness, vision deficiencies, gastric problems, anaemia, neurological problems, degeneration of spinal cord and nerve fibers etc (Pravakar & Roy,2009). Keeping these point in mind vitamin A,D,B₅ and B₁₂ parameters were selected for estimation in *B.bengalensis*. The present estimated result ensured, about the presence of vitamin A ,D, B₅ and B₁₂ at significant level in *B. bengalensis* (p < 0.05) (Table 15).

It was noticed that the estimated value of vitamin A in the fresh water garden snail, *Helix aspersa* was 5.462 \pm 0.05 mg/100 g (Cagiltay *et.al*, 2011). Where as in fresh water bivalves, *Lamellidens marginalis* the amount of vitamin A was reported 87.60 \pm 0.89 µg (Haldar *et.al*,2014). In other experiments the detected vitamin A,D,B₁₂ were scored highest 14.40 IU, 200 IU and 0.42µg/g respectively, in marine clam, *Meretrix casta* (Chemnitz) from Cuddalore and Parangipettai Coast, (South East Coast of India) (Srilatha *et.al*,2013). It was also studied that marine black mussel (*Mytilus galloprovincialis*) contained vitamin A and D₃ in the amount of 99.7 \pm 7.3 µg/100 gm and 14.8 \pm 1.0 µg/100 gm respectively (Merdzhanova *et.al.*,2014). Alongside, the average value of vitamin A, B₅, B₁₂ in pacific marine oysters were observed 270 IU, 0.5mg and 16 µg respectively.² While in squid the average value of vitamin A, B₅, B₁₂ were 10 IU, 0.5

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It was reported that the mode of function of vitamin A in two levels in the body. The first is in the visual cycle in the retina of the eye; the second is to keep the body tissues systemic towards maintain growth and the soundness of cells. The growth and differentiation of epithelial cells throughout the body are especially affected by vitamin A deficiency (VAD). Beside these, vitamin A plays a great role in growth and development, maintenance of epithelial cellular integrity, immune function, reproduction etc (FAO, 2001). The required amount of vitamin A for an adult was reported 270 μ g/day (FAO,2001).

While daily requirement of vitamin D was mentioned 5 μ g/day (FAO,2001). Vitamin D associated with normal mineralization and strengthening of bone, which prevents bone deformities and other bone related diseases, muscle contraction, nerve conduction and general cellular function in all cells of the body. It also maintain Ca and PO₄ level in blood (FAO,2001).

Pantothenate or vitamin B_5 (included in vitamin B complex), is a water-soluble vitamin. For many animals, pantothenic acid is an essential nutrient. Animals require pantothenic acid to synthesize coenzyme-A (CoA), as well as to synthesize and metabolize proteins, carbohydrates, and fats.³ Vitamin B_5 was reported to play great role in the breakdown of fats and carbohydrates for energy, also important in maintaining a healthy digestive tract. It was noticed that B_5 helped in speed wound healing, especially following surgery. This might be particularly true if vitamin B_5 is combined with vitamin

C also, that will be helpful to cure rheumatoid arthritis but more study in this regards, was reported to be required.⁴ The requirement of vitamin B_{5} , was reported for an adult was 5 mg/day (FAO,2001).

Vitamin B₁₂ (included in vitamin B complex), in food was reported to bind with proteins and released from the proteins by the action of a high concentration of hydrochloric acid present in the stomach. Where free form of the vitamin, which is immediately bound to a mixture of glycoproteins secreted by the stomach and salivary glands. This process ultimately enables its active absorption at the lower end of the small intestine, by phagocytosis process of specific ileal receptors (Weir and Scott, 1999). It was reported, as plants do not synthesize vitamin B₁₂, therefore, individuals who consume diets completely free of animal products (vegetarian diet) faced risk of vitamin B_{12} deficiency. Mal-absorption of vitamin B₁₂ resulted pernicious anaemia (Weir and Scott, 1999 and Chanarin, 1979), atrophic gastritis (due to bacterial overgrowth in the stomach and intestine reduced the chance B_{12} absorption), irreversible damage, especially to the brain and nervous system, fatigue, depression, and poor memory (ODSNIH,2011), mania and psychosis etc (Masalha et.al., 2001 and Sethi, 2005). The required level of safe intake of vitamin B_{12} was reported 2.4 to 2.8 μ g/day (FAO,2001). So here, it was assumed that the B. bengalensis indicated its good flesh value and the percentage of stated vitamin it contained at satisfactory level as per human requirement. Along with it can be also noted that the dependency of people (mainly rural people) on *B. bengalensis* due to get rid from several diseases, were not insignificant.

8.5 Fluctuation of nutritional parameters of *B. bengalensis* with the seasonal change

Hence the present study was carried out to evaluate the changes in nutritional value, regarding the seasonal variation of *Bellamya bengalensis*. The present investigation also represented about this gastropods, exhibit maximum protein, carbohydrate and lipid content in Pre-Monsoon season (February to May). While those parameters were rather minimum at Post-Monsoon seasonal period (October to January). This variation in biochemical parameters may be primarily assumed about the availability of favorable environmental conditions, which leaded maturation and gonadal development (Table 16).

It was noticed that, Pre-Monsoon season (February to May) indicated to abundant maximum percentage of mainly protein than Monsoon (June to September) and Post-Monsoon season (October to January), which can be correlated with highest body activities of this snail during this season. Accumulation of protein in this fresh water snail during the Pre-Monsoon season corresponds with the proliferation of gonads (John, 1980).Percentage of these constituents also increases with the maturation of gonads (Ansell,1972). According to Lee (1986) this maximum and minimum level of protein correspond with the development/ spawning and regression/ resting phases, respectively. This could be mainly because of increase food availability and secondly it happened just prior to the spawning period. This increased protein content may be a mechanism of maturation of gonad and storage of reserves to meet spawning requirements (Nagabhushanam et.al., 1978 and Wafer et.al., 1976). Compare to all, low protein value recorded in Post-Monsoon might be mainly due to increased rate of ammonia excretion and also for spawning activity especially during post spawning season which were in conformity with earlier reports (Gabbot and Bayne, 1973). And this minimum protein

values in Post-monsoon season, which may be due to sedentary life without much activities.

Though it was observed, in the later part of Pre-monsoon (May) season the percentage of the protein became to some extent indicated its reduction tend. Nevertheless, at the onset of the Monson season (June), it was seen to increase but at the later part of the monsoon (September), it showed slow decreasing tendency in the concentrations of protein, which extended up to the middle of end part of the Post monsoon (starting of January) season. From the middle of end part of the Post monsoon season (starting of January) the concentration of mainly protein (along with fat and carbohydrate) notice to keep it slow increasing trend, throughout the experimental period. *B. bengalensis* is a life barer snail and it released tinny snail juvenile (instead of eggs). The batch wise gonadal developmental process was noticed in its uterine chamber (which will be discussed in details at the Capter III) (Fig. 15).

Though spawning occurs mainly during the Pre-Monsoon and Monsoon season in *B. bengalensis*, which was represented by the large depletion in the protein and lipid contents. Soon after spawning, within a short period of relative inactivity, during which the un-spawned sex cells were reabsorbed, the mussels began to accumulate and store carbohydrates in their tissues. Accumulation and depletion of these stored reserves in this fresh water snail also depends on the environmental influences on metabolic activities, with the quantity and quality of available food (Ansell,1972) and this was well described by several authors (De Zwaan,1972;Dare *et. al*,1975 and Gabbott,1975).

Generally, carbohydrates which stored as glycogen was attributed as the primary energy store house in this snail (Banye,1976 and Garbbott,1983). This relative amount of stored glycogen in this snail tissue was also considered as a good indicator of body condition (Galtsoff, 1964 and Walne, 1970). This glycogen remained at a high level until the beginning of proliferation of gonads. The amount of carbohydrates present in the muscle tissues of *B. bengalensis* might be closely linked with the food availability and gonadal development. Carbohydrate was found to be at maximum during Pre-monsoon season, which shows the development of gonads to attain maturation. During the rapid proliferation of these gonads, the reserve supply was used, and by the end of the reproductive cycle the amount of carbohydrate was at a minimum. Not only that this glycogen content in this snail, was observed to decline, might be due to starvation, reproductive stages, change in environmental conditions, low metabolic rate etc (Fig. 16).

Same as carbohydrate (glycogen), lipid also acted as the source of energy (Shigmates et.al., 1959, Beukema, 1979, Beninger et.al., 1984 and Chourpagar et.al., 2011) and also played a great role in the significant increases in egg diameter, egg lipid content, hatching rate and larval survival etc (Uriarte et.al., 2004). Lipid variation was related to gamete development with the highest levels of lipids accumulation during the gonadal ripening (Fig. 17). But in and after monsoon months as the temperature and other abiotic parameters changed, lipids also decreases (Shafakatullah and Krishnamoorthy, 2014). The increase in lipid contents may be due to the lipogenesis occurring in the ovary for production of gametes. In the present study this declination of the lipid content (starting from Monsoon up to Post-Monsoon season) was indicated due vegetation, environmental temperature, abiotic and biotic factors of the water. Most importantly diet may be played a major role in it (Shafakatullah and Krishnamoorthy, 2014). In the Pre-Monsoon season due to exposure of foot at the increasing temperature, the lipid molecules may be deposited in large amount in the body tissue. During the maturation and breeding (mainly February to August), this

concentration of lipid exhausted in gonadal follicles and time of spawning. Hongwei *et al.*, (2009), observed protein and lipid content increased in association with the gametogenesis in the female molluscan gonads. The present study revealed that in terms of energy conservation, the organism would be expected to make compensatory adjustments to both the components of energy gain and energy loss in the fate of changes in the environmental conditions (Vedpathak, 1989).

The nutrients such as protein, carbohydrate and lipids were observed to vary seasonally. These changes were generally related to the reproductive cycle. Several works had been also carried out, in season wise variation of biochemical parameters. of L.jenkinsianus was found to be, proteins (40.8%-61.2%), lipids (4.7%-8.6%), carbohydrate (15.39% -40.7%) and ash content (10.3%-32.1%) of dry tissue weight. In Lamellidens.generosus protein (42%-62.4%), lipids (4.7%-8.7%), carbohydrate (19.0% -37.4%) and the ash content (9.7%-25.6%) (Shafakatullah et.al., 2013). This similar characteristics (season wise variation of biochemical parameters) had been also observed within the different group molluses, such as Anomalocardia squamosa (Morton, 1978), Donax trunculus (Ramon et.al., 1995), Lyropecten (Nodipecten) nodosus (Lodeiros et.al., 2001), Mercenaria mercenaria (Peterson et.al., 1986), Placopecten magellancius (MacDonald and Thompson, 1986), Parreysia spp (Shafakatullah et.al., 2014), Lamellidens (Shaikh,2011),Parreysia marginalis corrugate (Malathi and Thippeswamy, 2013), Donax scortum (Singh et.al., 2012) etc.

In this present study, seasonal variation in the levels of protein, carbohydrate and lipids content due to storage and utilization of the few organic constituents which was closely linked to complex interaction between food supply, temperature, other environmental factors and also between growth and reproductive cycle (Lodeiros,2001).