CHAPTER II

LITERATURE REVIEW

Penaeus monodon

The giant tiger prawn, Penaeus monodon Fabricius, is one of the largest and most commercially important species among penaeid prawns in the world. This species widely distributes throughout the greater part of the Indo-West Pacific region. Life history of P. monodon is classified into six phases: embryo, larva, juvenile, adolescent, subadult and adult. Its color is dark brown to blackish. Their food consists mainly of small crustaceans, mollusks and annelids. The adult is a predator of slow moving benthic macroinvertibrates or opportunity in feeding behavior. The tiger prawn is relatively eurythermal and euryhaline and grows rapidly to a marketable size. Its life span is approximately one and a half to two years (Motoh, 1979).

Larval development of P. monodon

Development of larval stages of P. monodon consists of 6 nauplius, 3 protozoea, 3 mysis and 3-4 megalopa substages or postlarvae (Fig1). Time of development for each stage requires about 1.5 days, 5 days, 4-5

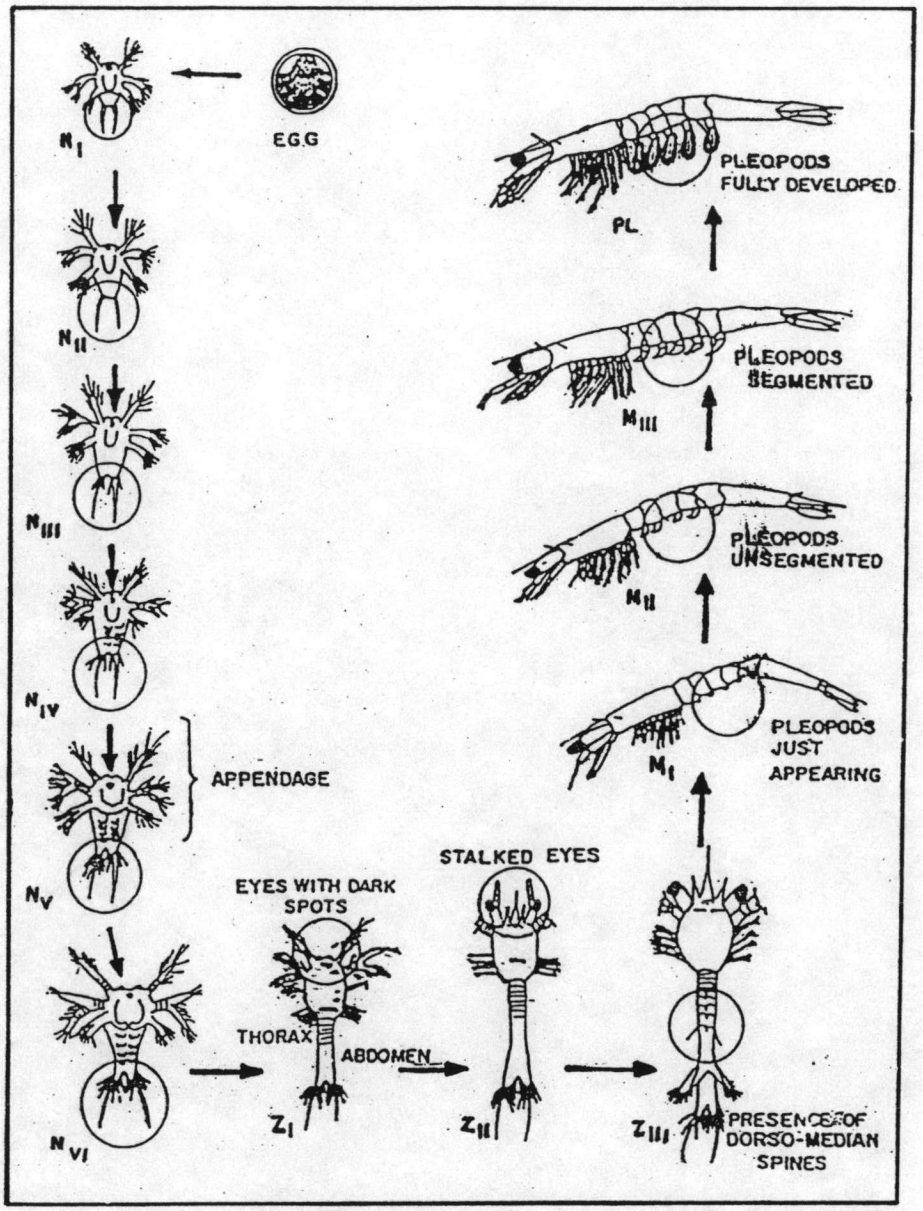


Figure 1 Larval stages of Penaeus monodon (Anonymous, 1988)

N = Nauplius stage, Z = Zoea stage, M = Mysis stage

and PL = Postlarva

days and 6-15 days, respectively (Motoh, 1979, 1984). Larvae exhibit planktonic offshore behavior with antennal propulsion for swimming in nauplius, antennal and thoracic propulsion in mysis and abdominal propulsion in postlarvae. Normally, there is no feeding requirement in the nauplius stage because they utilize yolk granule within their body. Phytoplankton, such as Chaetoceros sp., Skeletonema sp. and Tetraselmis sp. are introduced from the first zoea to the second mysis. Zooplankton such as, rotifer and Artemia sp. are fed from the third mysis to postlarva (Motoh, 1979). Sometimes, it is necessary to provide artificial food to the larvae when natural food may be absent or quality of natural diet is poor.

Artificial feed for shrimp larvae

Many natural foods, for example phytoplankton and zooplankton, are suitable in size and nutrition for shrimp larvae but their productions depend on environmental factors, especially light and temperature. It is quite difficult to control environmental factors in mass culture of algae. It also needs space for culture and stock maintenance. Artificial food is interesting. It is easy to produce, quality control and storage. With an aim to improve the knowledge on qualitative nutritional requirements of larval stages of shrimp, several investigators have tried to develop purified particulate diets which can be used as standard reference diets in nutritional studies. Attempts in

this respect for crustaceans have been made by Kanazawa and Castell in 1990 (Lavens et al., 1992).

Akiyama et al. (1988) determined the apparent dry matter digestibility (ADMD), apparent protein digestibility (APD) and apparent amino acid digestibility (AAAD) of various ingredients for shrimp. From ingredients evaluations, it showed that ADMD and APD of casein were higher than soy protein, fish meal, soybean meal, shrimp meal, squid meal and rice bran. ADMD of the purified feedstuffs was observed as being more efficiently digested compared to the practical feedstuffs. The difference was also observed in the APD and AAAD. This indicates that proteins are more readily digested in the purified form.

Results from Sutjaritvongsanon (1984) showed that zoea of P. japonicus can develop to postlarvae within 9-10 days with 84% survival rate when fed with suitable microparticulated diet.

Microparticulated artificial diets have been tested as partial or complete replacements for live algae or zooplankton for maricultured animals (Chu et al., 1987; Jones, Kurmaly and Arshad, 1987; Laing, 1987). The rationate is that the nutritional composition can be controlled. Feed costs and maintenance might be reduced. Feeding trials of juvenile oysters and clams showed that partial replacement of Chaetoceros calcitrans with an artificial diet did not significantly reduce the animals' growth rate (Laing, 1987). In

prawn larvae, an artificial diet successfully promoted growth through to postlarval stage (Jones et al., 1987) but their survival rate was lower and more variable. It suggests that the artificial diet was not completely substituted to live algae or Artemia sp.

The continuing effort to improve animal performance (growth, survival and feed efficiency) in aquaculture has stimulated a search for new additives for shrimp feeds. There is a real need to study the role of certain micronutrients and vitamins, especially astaxanthin on aquatic animal health. Catacutan and Cruz (1989) reported that P. monodon fed no vitamin diet had the poorest growth rate. The absence of vitamins in the diet has serious effects on growth and survival of prawns. Astaxanthin was reported to gain some beneficial effects on P. monodon maturation and larval performance. The dietary astaxanthin could enhance gonad maturation and spawning, produce significantly bigger egg diameter and lower mortality rate of female broodstocks (Menasveta et al., 1993).

Astaxanthin

Carotenoids are exogenously derived from isoprenoid compounds which are responsible for pigmentation in nature and have essential biological functions in animals (Latscha, 1989). They also impart attractive pigmentation to many farmed animals. Since animals are lack of ability to

synthesize carotenoids, the pigment must be supplemented to feeds. Usually, carotenoids at considerable expense to the farmer (10% to 15% of the total feeds costs) (Johnson and An, 1991).

Astaxanthin (3,3'-dihydroxy-β, β-carotene, 4,4'-dione) is the principle carotenoid pigment in crustaceans and salmonids, various bird including flamingoes, scarlet ibis and many other organisms (Kobayashi et al., 1992A). About 90% of the total pigment in the carapace of P. japonicus and Pandalus borealis is astaxanthin (Latscha, 1990). This carotenoid imparts distinctive orange-red coloration to the animals and contributes to consumer appeal in the marketplace. Since astaxanthin has been shown to posses higher antioxidative activity than β-carotene and α-tocopherol, it could be supplied as a food colorant or in medical use (Tjahjono et al., 1994).

Astaxanthin is also the preferred pigment in aquaculture since it is deposited more efficiently than other carotenoids in salmon and trout (Bjerkeng, Storebakken and Jensen, 1990; Foss et al., 1984; Torrissen, 1984). Most fish farmers used synthetic astaxanthin and canthaxanthin in feeds at levels of 35 to 75 mg/kg dry feed, but synthetics are expensive and have not been approved by U.S. Food and Drug Administration (FDA) for using in salmonid rations. Furthermore, the synthetic formulations may contain unnatural configurational and *cis-trans* isomers and carotenoid-like compounds, that are not active form for animals. All these factors have

contributed to the interest to find natural sources of carotenoids (Johnson and An, 1991).

Chemical Properties of Astaxanthin

Astaxanthin is an oxycarotenoid with a molecular formula, $C_{40}H_{52}O_4$ and molecular weight 596.86. Astaxanthin was first chemically identified by Kuhn and Sorensen base on Karrer's earlier recognition of astacene (Johnson and An, 1991). Davies and Weedon (1960) later confirmed the structure by partial synthesis of its derivative astacene from canthaxanthin. The total synthesis of optically pure astaxanthin from racemic intermediates has been accomplished by researchers at F. Hoffmann-LaRoche, Switzerland (Johnson and An, 1991). Isolated crystalline astaxanthin has the appearance of a fine, dark violet-brown powder. Its melting point is approximately 224 ^{o}C. It is insoluble in aqueous solution and most organic solvents but can be dissolved at room temperature in non polar solvents. Because carotenoids contain a long conjugated double bond system, they are less stable than other isoprenoids and precautions must be taken to avoid artifacts and destruction of the pigments. Light, heat, acid and oxygen are particularly detrimental to carotenoids and enzymatic destruction also can occur during extraction from biological samples (Stanier, Kunizawa and Cohen Bazire, 1971).

High-performance liquid chromatography (HPLC) is useful for detecting *cis*-isomers of astaxanthin, which generally elute later from the

column. All-*trans* natural astaxanthin and other carotenoids are readily isomerized to *cis-trans* mixtures, especially the 9-*cis* and 13-*cis* unhindered isomers and precautions must be taken to avoid their formation. *Cis*-isomers of carotenoids are generally considered to be unnatural artifacts. However, *cis-trans* isomerism of vitamin A (retinol) occurs naturally in the retina and the 11-*cis* retinol isomer is thermodynamically less stable and energy is required for the endergonic transformation of vitamin A to 11-*cis*-retinol (Johnson and An, 1991).

Astaxanthin has two asymmetric carbon atoms at the 3 and 3' position and can exist in four configurations, including the identical enantiomers (3S, 3'S; 3R, 3'R) and meso forms (3R,3'S; 3'R, 3S) (Foss et al., 1984; Storebakken and No, 1992). Chemical synthesis from racemic precursors gives equal mixtures of the configurational isomers. The isomers can be separated by reacting (-)-camphanic diasteriomeric diesters by HPLC and by TLC on silica gel (Foss et al., 1984). Configurations of astaxanthin are shown as Fig 2.

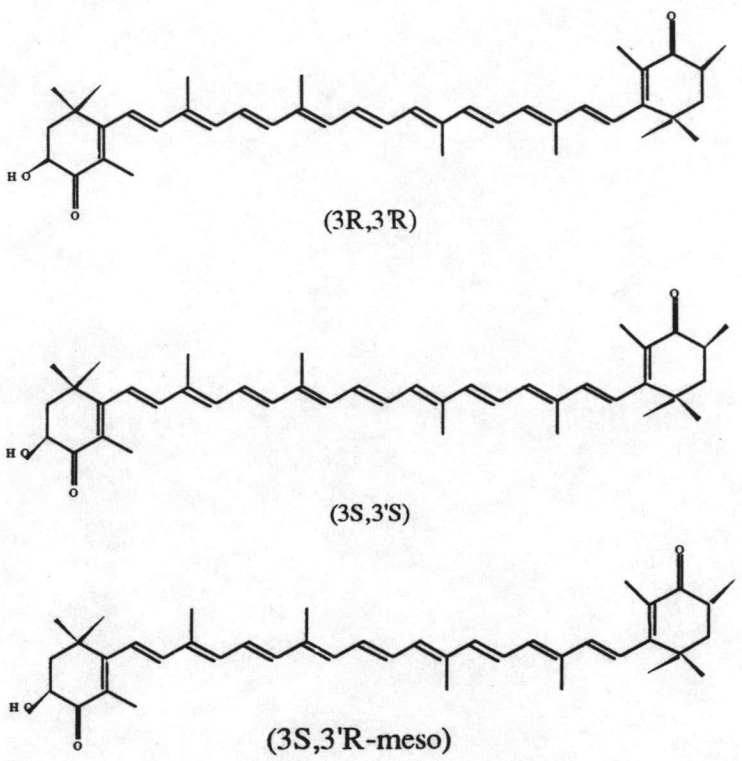


Figure 2 Three configurations of astaxanthin (Johnson and An, 1991)

Astaxanthin in crustacea

Astaxanthin exists in three forms in the crustacea ; 1) as free form, 2) as astaxanthin esterified to long-chain fatty acids and 3) as astaxanthin attached to protein as carotenoprotein. All three types have been detected in many species. The free pigments appear to accumulate in the hepatopancreas

while the carotenoprotein have been observed in eggs, ovaries, retina, hypodermis, cuticle (chitin), gut and hepatopancreas. The carotenes and esterified astaxanthin appear to be storage forms and dissolved in lipids in the hepatopancreas from which they are transferred to other sites in the body by haemolymph (Ghidalia, 1985).

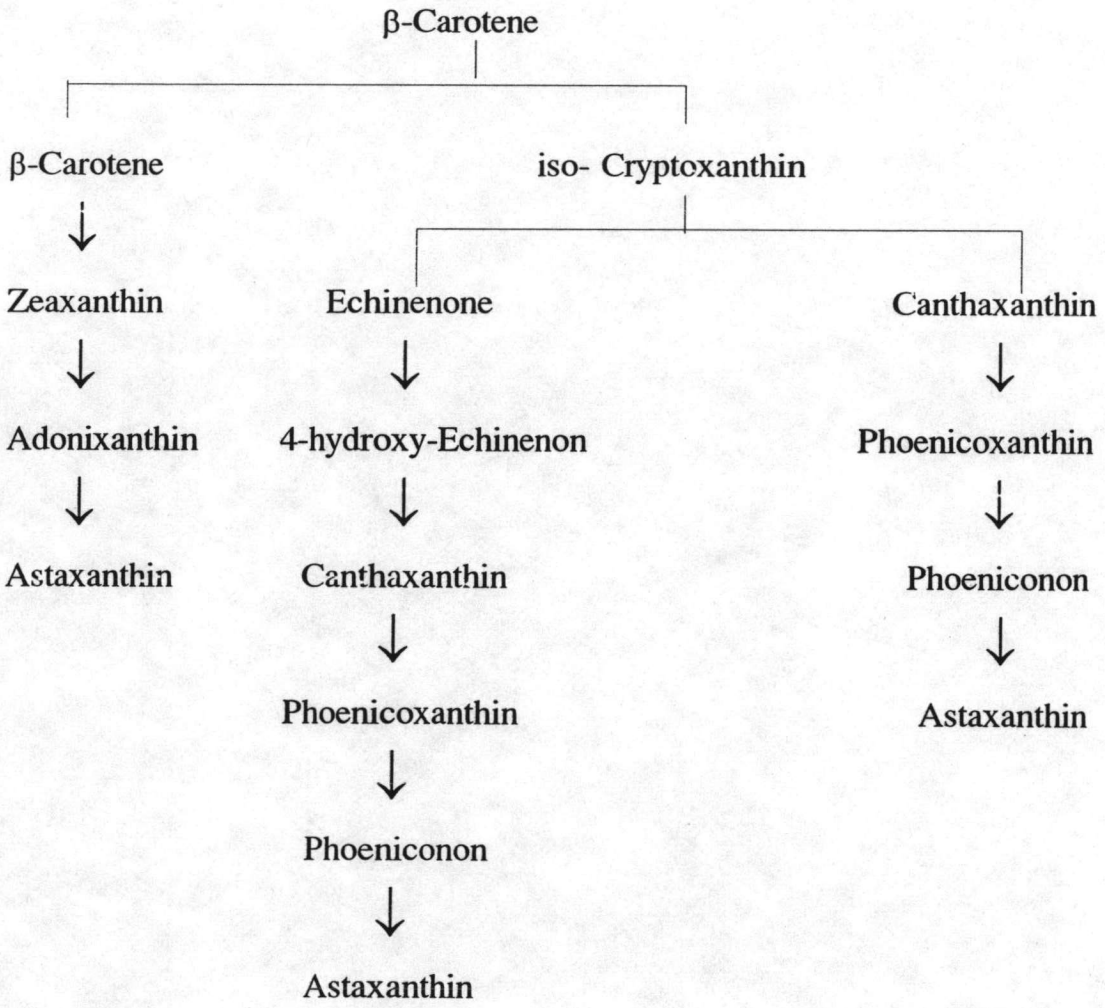
It is found that the shrimp Penaeus sp. is able to convert dietary carotenoids to astaxanthin. Like most crustaceans, they belong to the class of aquatic animals that can oxidize carotenoids at the 3,3' as well as the 4,4' position, regardless of whether the β-end groups are primarily oxidized in one of the two position or not at all. This implies that shrimp, crab and lobster can convert β,β-carotene, zeaxanthin (β,β-carotene-3,3'-diol) and canthaxanthin (β,β-carotene-4,4'-dione) to astaxanthin, but they can absorb astaxanthin as well (Schiedt, Bischof and Glinz, 1993). Astaxanthin also has been reported to mask the effects of "Blue shell syndrome" (sky blue color instead of the normal brown-black, shells sometimes soft, thin and rough surface and absense of intense red color after cooking) (Akiyama and Dominy, 1992). Proposed carotenoid functions in crustaceans and possible pathway from β-carotene to astaxanthin are shown in Table 1 and Fig 3, respectively.

Table 1 Proposed carotenoid functions in crustaceas

Proposed carotenoid function in Crustacea
Light perception
Electron acceptor
Protection :
Chromatic adaptation
Eggs from radiation
High temperatures
Reducing reflectivity
Masking luminescence of prey in stomach
Gut wall against digestive enzymes
Stabilization of protein
Stabilization of chitin
Transfer of pigments (carotenoprotein)
Chemoreception in antennae

Source: Goodwin and Jamikorn, 1954

Figure 3 Metabolic pathway of carotenoids in prawn, crab and lobster



Sources : Katayama, Hirata and Chichester, 1971; Latscha, 1989; Tanaka et al., 1975.

Astaxanthin sources for salmonids and crustaceans

According to Johnson and An (1991), astaxanthin for farmed animals came from different sources such as:

1. Synthetic astaxanthin

F. Hoffmann-La Roche, Basal Switzerland accomplished to synthesize of *trans*-astaxanthin which is marketed as "Carophyll pink" containing 8% astaxanthin (Latscha, 1989). Synthetic astaxanthin is presently the principal source used in feeds. An important precursor for the synthesis of astaxanthin is (S)-3-acetoxy-4-oxo-β-ionone which can be obtained by asymmetric hydrolysis of the (R)-terpene alcohol acetates by various organisms.

2. Crustaceans and crustacea byproducts

Shrimp (Pandalus borealis) wastes have been used traditionally as natural pigment sources for trout and salmon (Guillou et al., 1995). Carotenoid levels in most crustaceans are usually quite low and satisfactory pigmentation requires the addition of 10 to 25 % by weight of the chitinous extract to the bulk diet. Crustaceans wastes have high level of ash, chitin, moisture and low levels of protein and other nutrients that limit their usefulness in animal feed.

3. Algae

Certain green algae in the subphylum Chlorophyceae possess astaxanthin as their secondary carotenoid (Bubrick, 1991; Boussiba and Vonshak, 1991). Depending on the method and control of culture, very high levels of astaxanthin accumulation in Haematococcus pluvialis (0.5-2.0% astaxanthin in dry weight basis). Most of the astaxanthin (87%) is esterified (Kakizono et al, 1992), which may affect its deposition and metabolism in some animals. Low deposition of astaxanthin in salmon fed algae obtained by Kvalheim and Knutsen (1985) suggested that astaxanthin was present principally as esters, however, poor deposition caused solely by esterification was not confirmed by others. The predominant configurational isomer in H. pluvialis was shown to be 3S, 3'S. Moreover, astaxanthin can be produced by Chlamydomonas sp. and Neochloris wimeri (Bubrick, 1991).

4. Yeast

The colonies of yeast Phaffia rhodozyma can produce carotenoid pigments mainly astaxanthin. Andrews and Starr (1976) determined configuration of yeast astaxanthin and made unexpected discovery that the predominant isomer was 3R, 3'R, the opposite of his earlier finding of 3S, 3'S in lobster. Wild strains of P. rhodozyma so far isolated containing up to 500 μg total carotenoid/g dry weight (or 0.02 to 0.03%) of which 40 to 95% is astaxanthin. The content of astaxanthin varies substantially depending on strain and method of culture.

5. Other microorganisms

Some bacteria, including Mycobacterium lacticola, Brevibacterium sp. and fungi in the genus Peniophora have been reported to contain astaxanthin (Droop, 1955). Carotenoid levels of these microorganisms are low and growth rates are slow. Fermentation development has not been pursued. The industrially important xanthophyll, canthaxanthin and zeaxanthin are produced by Brevibacterium sp. and Flavobacterium sp. but the productivity is too low for commercial.

Haematococcus pluvialis Biology

1. Taxonomy

Division	Chlorophyta
Class	Chlorophyceae
Order	Volvocales
Family	Haematococcaceae
Genus	Haematococcus sp.
Species	H. pluvialis

Sources : Chapman, 1962; Smith, 1950

2. Characteristics and reproduction of H. pluvialis

Motile cells of Haematococcus sp. are solitary, biflagellate and enclosed by a wall that is broadly ellipsoid to ovoid. The protoplast lies some distance inward from the wall and connects with delicated strands of cytoplasm. The intervening space between the wall and the protoplast is filled with a watery gelatinous substance. There are two flagella at the anterior end of a wall and the portion of each flagellum between the wall and protoplast lies with a gelatinous (Smith, 1950). Droop (1955) reported that two strains of H. pluvialis can utilize organic nitrogen and acetate to grow heterotrophically in darkness. Under stress condition, the cells transform into nonmotile, brick-red akinetes. The red color is called haematochrome or astaxanthin (Pringsheim, 1966). H. pluvialis is preferred to use for production of astaxanthin because of its proficient and rapid growth (Spencer, 1989). Fractions of carotenoids produced by H. pluvialis are shown in Table 2.

Asexual reproductions of Haematococcus sp. take place by division of free-swimming cells into two or four daughter cells (macrozoospores). More frequently a cell develops into an akinete. When an akinete germinates, its protoplast divides into 4, 8 or 16 zoospores that are liberated by a sac-like swelling of the akinete wall division of an akinete's protoplast resulting in a formation of aplanospores instead of zoospores. Aplanospores are liberated in the same manner as zoospores (Smith, 1950). Akinetes which have been

subjected to adverse condition, such as cold, rapid drying or starvation, usually have the protoplast dividing into 32 or 64 biflagellate swarmers. These are sometimes called "microzoospores", the gametes which cannot develop parthenogenetically into vegetative cells. Gametes may also be formed by repeating division of the protoplast of a vegetative cell (Bold and Wynne, 1978; Chapman, 1962). Life cycle of H. pluvialis is shown in Fig 4.

Table 2 Fractions of the carotenoids produced by H. pluvialis

Carotenoids (%)	H. pluvialis strains			
	CCAP 34/7 [1]	MUR 145 [1]	NIES144 (base) [2]	NIES144 (modified.) [2]
β- carotene	5	5	n.d.	n.d.
Echinenone	3	4	n.d.	n.d.
Astaxanthin:				
diester	39	34	10	20
monoester	49	46	74	69
free form	1	1	n.d.	n.d.
Canthaxanthin	2	4	n.d.	n.d.
Adonirubin ester	2	-	6	9
Lutein	1	6	9	1

Remark : n.d. means not detectable

[1] Harker, M. 1995., [2] Kobayashi et al., 1992B

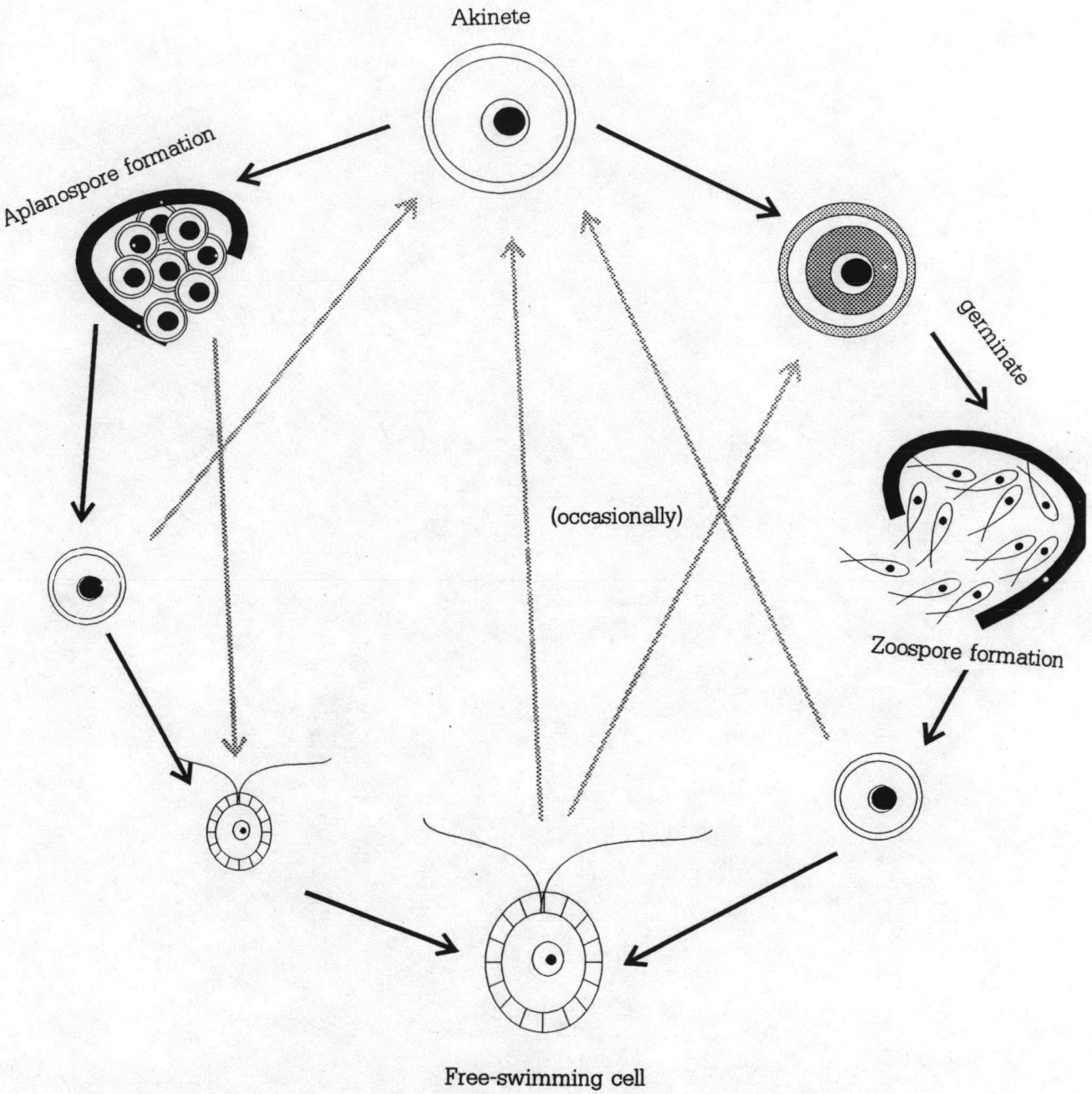


Figure 4 Life cycle of Haematococcus pluvialis (Chapman, 1962)

3. Environmental regulation of astaxanthin biosynthesis in Haematococcus sp.

Astaxanthin production in Haematococcus sp. is regulated by several environmental factors, including light, aeration, nutrition and other factors as follow (Johnson and An, 1991);

3.1 Kinetics of growth and pigment formation

Astaxanthin content varies significantly during the life cycle of Haematococcus sp. Astaxanthin is not found in growing vegetative cells but is formed during encystment of the alga.

3.2 Light

Light is important for the regulation of carotenogenesis in a wide variety of organisms. Carotenes are well known to react with radicals and recently Terao (1989) show that oxygenated carotenoids, particularly canthaxanthin and astaxanthin, also react efficiently with oxygen radicals. Light is known to stimulate astaxanthin formation in H. pluvialis (Boussiba and Vonshak, 1991; Kakizono et al., 1992; Pringsheim, 1966). Droop (1955) calculated that astaxanthin synthesis was seven times higher in light. Goodwin and Jamikorn (1954) showed that H. pluvialis cultures placed in the dark decreased in carotenoid content and did not change from green to red or brown phases.

3.3 Temperature

Temperature has been reported to have little impact on carotenoid formation in green algae.

3.4 Nitrogen and carbon

In the Chlorophyceae, nitrogen limitation is a key factor for accumulation of astaxanthin (Kobayashi et al., 1991B). Acetate and glycine were demonstrated to stimulate astaxanthin formation in H. pluvialis, whereas other nitrogen and carbon sources delayed the onset of production. Hence, the quality and availability of carbon and nitrogen are critical for carotenogenesis in H. pluvialis .

3.5 Other nutrients

Limitation of certain micronutrients, including manganese, iron and sulfate enhances secondary carotenoid formation in some microalgae. Moreover, fluoride stimulates carotenogenesis in certain fungi.