THE APPLICATION OF THE BRINE SHRIMP *ARTEMIA SALINA* FOR DELIVERY OF BIOLOGICAL MOLECULES IN AQUACULTURE

NUR IDAYU BINTI ADNAN

THIS DISSERTATION SUBMITTED TO MEET
TERMS OF OBTAINING
BACHELOR OF SCIENCE WITH
HONOURS

BIOTECHNOLOGY PROGRAMME
FACULTY OF SCIENCE AND NATURAL RESOURCES
UNIVERSITY MALAYSIA SABAH

2014

ARKIB

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS

JUDUL: THE APPLICATION OF THE BRINE SHRIMP ARTEMIA SALINA FOR DELIVERY OF BIOLOGICAL MOLECULES IN AQUACULTURE

IJAZAH: IJAZAH SARJANA MUDA BIOTEKNOLOGI

SAYA: NUR IDAYU BINTI ADNAN (HURUF BESAR)

SESI PENGAJIAN: 2011/2014

Mengaku membenarkan tesis *(LPSM/Sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis adalah hakmilik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

[ ] SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)

[ ] TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana Penyelidikan dijalankan)

[/] TIDAK TERHAD

Disahkan oleh: NURULAIN BINTI ISMAIL
LIBRARIAN
UNIVERSITI MALAYSIA SABAH

(TANDATANGAN PENULIS)

(TANDATANGAN PUSTAKAWAN)

Alamat tetap: NO-13, JLN M15, TMN MERBOK, BUKIT KATIL, 75450, MELAKA

DR KENNETH RODRIGUES
NAMA PENYELIA

Tarikh: 26 JUN 2014

Tarikh: 27/6/14

Catatan :- * Potong yang tidak berkenaan.
*Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.
*Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana Secara penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM)

DECLARATION

I declare that this is my own work excepts for excerpts and summaries of each have been duly acknowledged.

NUR IDAYU BINTI ADNAN

(BS 11110480)

26 JUNE 2014

VERIFICATION

SIGNATURE

SUPERVISOR

(DR KENNETH F. RODRIGUES)

APPRECIATION

I would like to thank Dr. Kenneth F. Rodrigues as my supervisor and he has provided advice and helped me to conduct this project as effectively as possible. I would also like to thank all of my lecturers especially my co-supervisors Dr. Ivy Wong Nyet Kui, Dr. Zaleha Aziz, Dr. Jualang Azlan Gansau, Dr. Ching Khim Phin, and Miss Hartinie Marbawi who took the trouble to give their knowledge and provide guidance and support to me during my studies at Universiti Malaysia Sabah.

Next, thanks are given to Mr. Fernandes Opook since has been equally helpful and assisted me from the beginning till the end of my research. He had guided me and shared knowledge to me for my conduct on this research.

I would also like to give an appreciation towards my fellow friends who always support me to carry my study no matter how harsh the condition become especially Yushahfira Binti Akul, Afiqah Shafify Binti Amran, Rozalia Binti Geoffery, Eryati Binti Derman and Chua Chuen Yang

The most special thank goes to my family especially my parents, Adnan Bin Mohd Nor and Ruhaya Binti Abdul Rahman. And I thank Allah for all these wonderful people that He granted me with.

NUR IDAYU BINTI ADNAN
BS1110480

THE APPLICATION OF THE BRINE SHRIMP ARTEMIA SALINA FOR DELIVERY OF BIOLOGICAL MOLECULES IN AQUACULTURE

ABSTRACT

The brine shrimp *Artemia salina* (Linn, 1758) is generally used as live feed in the aquaculture industry. The relative ease of propagation, tolerance to extreme salinity and high protein content make *Artemia salina* ideal for feeding marine organisms at the post-larval stage. The ability of *Artemia* to feed on microbes makes them amenable to application for the delivery of recombinant protein molecules and recombinant DNA into marine organisms. This investigation focuses on the application of the *Artemia* for the delivery of recombinant protein produced in *Escherichia coli* (BL21) using a Green Fluorescent Protein (GFP) reporter as well as a recombinant plasmid PMCS2.1-ENTH-GFP which contains eukaryotic promoter and terminator elements. The findings of this study will facilitate our understanding of the process by which *Artemia* can uptake recombinant proteins and express plasmid DNA constructs containing recombinant protein coding genes. From this study, the *Artemia* has been able to retain the GFP produced by the *Escherichia coli* (BL21) which make it suitable for the protein retention and delivery to aquaculture organisms but the *Artemia* unable to transcribe the gene from plasmid PMCS2.1-ENTH-GFP which has been fed orally. However, the *Artemia* could express the protein from plasmid PMCS2.1-ENTH-GFP using biolistic gun method in transformation. Therefore, from this experiment, it can be conclude that *Artemia* besides having unique characteristics, it can be manipulated for the delivery of biological molecules in aquaculture.

APLIKASI UDANG AIR GARAM *ARTEMIA SALINA* UNTUK PENGHANTARAN MOLEKUL DALAM AKUAKULTUR

ABSTRAK

Udang air garam, Artemia salina (Linn, 1758) biasanya digunakan sebagai makanan hidup dalam industry akuakultur. Pada peringkat pasca larval, Artemia salina sangat ideal untuk digunakan sebagai makanan kepada organisma laut disebabkan pembiakannya yang mudah, mampu bertoleransi terhadap air yg berkemasinan melampau, dan mengandungi kandungan protein yang tinggi. Artemia salina mempunyai kebolehan menyerap mikroorganisma sebagai makanan membolehkan ia digunakan sebagai aplikasi untuk mengangkut molekul protein rekombinan dan DNA rekombinan kepada hidupan laut. Penyiasatan ini berfokus terhadap aplikasi Artemia untuk menghantar molekul protein rekombinan yang dihasilkan di dalam bakteria Escherichia coli (BL21) menggunakan promoter Green Fluorescent Protein (GFP). Selain itu, ia juga bertujuan untuk mengkaji penghantaran molekul plasmid pDREAM2.1/MCS-ENTH-GFP yang mengandungi promoter dan terminater eukarya. Penemuan kajian ini dapat membantu dalam memahami proses pengambilan protin rekombinan oleh Artemia dan menzahirkan koding protin rekombinan yang dibina di dalam DNA plasmid. Daripada kajian ini, Artemia mampu menahan protin yang dihasilkan daripada E. coli (BL21) iaitu protin GFP yang mana sesuai untuk penampungan protin dan juga sebagai penghantaran dalam bidang akuakultur. Akan tetapi, Artemia tidak dapat mengekspres gen daripada plasmid PMCS2.1-ENTH-GFP yang telah diberi sebagai bahan makanan. Manakala, Artemia dapat mengekspres gen daripada plasmid PMCS2.1-ENTH-GFP melalui cara pistol biolistic yang dilakukan dalam proses transformasi. Sebagai kesimpulannya, Artemia selain mempunyai ciri-ciri yang unik, ia dapat dimanipulasi sebagai ajen penghantaran molekul biologi di dalam bidang akuakultur.

CONTENTS

		PAGES
DECLARATION		ii
VERIFICATION		iii
APPRECIATION		iv
ABSTRAK		v
ABSTRACT		vi
CONTENTS		vii
LIST OF TABLES		xi
LIST OF FIGURES		xii
LIST OF SYMBOLS		xiii
CHAPTER 1 INTRODUCTION		1
CHAPTER 2 LITERATURE REVIEW		
2.1	*The Brine Shrimp*	5
	2.1.1 Classification	6
	2.1.2 Natural Habitat	7
	2.1.3 Characteristics	8
	2.1.4 Life Cycle	10
2.2	Application	14
	2.2.1 Application in Aquaculture	14
	2.2.2 Other Application	15
2.3	ENTH-GFP	16
2.4	Plasmid	16
	2.4.1 pGS-21a	17
	2.4.2 pDREAM2.1/MCS	18

2.5 Bacterial Strains 19
2.5.1 *Escherichia coli* BL21 (DE3) 19
2.5.2 *Escherichia coli* (TOP10) 19

2.6 Techniques and Equipments 19
2.6.1 Genetic Recombinant 19
2.6.2 Biolistic Gun 20
2.6.3 Fluorescence Microscopy 21
2.6.4 Plasmid Preparation 21
2.6.5 SDS-PAGE 22
2.6.6 Restriction Enzyme 22
2.6.7 Experimental Design 23
2.6.8 Expected Result 24

CHAPTER 3 METHODOLOGY
3.1 Validation of plasmid DNA by using restriction enzyme method 25
3.1.1 Plasmid extraction 25
3.1.2 DNA and plasmid quantification 26
3.1.3 Restriction enzyme 27
3.1.4 Gel electrophoresis 27

3.2 Validation of protein by SDS-PAGE 28
3.2.1 Sample preparation 28
3.2.2 SDS-PAGE 28

3.3 Culture of cysts 29

3.4 Establishment of populations 29

3.5 Preparations for feeds (protein and plasmid purposes) 30

3.5.1 Culture of plasmid pGS21-a-ENTH-GFP in *Escherichia coli* BL21 (DE3) cells 30
3.5.2 Culture of plasmid pMCS2.1-ENTH-GFP in *Escherichia coli* (TOP10) cells 31

3.6 Cell washing 31

3.7 Feeding experiment 31

3.8 Sampling for visualization 31

3.9 Visualization using fluorescence microscopy 32
3.9.1 Microscope slide preparation 32
3.9.2 Visualization under fluorescence microscope 34

3.10 Transformation of cysts using biolistic gun 33

3.10.1 Plasmid extraction and purification 33
3.10.2 Sample (cysts) preparation 33
3.10.3 Coating washed microcarriers with DNA 33
3.10.4 Preparation for bombardment 34
3.10.5 System bombardment set up 34
3.10.6 Culture and visualization of the bombarded cysts 35

CHAPTER 4 RESULTS

4.1 Validation of protein produced by the *Escherichia coli* (BL21) containing plasmid pGS-21a-ENTH-GFP and pDREAM2.1/MCS-ENTH-GFP through SDS-PAGE. 37

4.2 Validation of plasmid pGS-21a-ENTH-GFP and pDREAM2.1/MCS-ENTH-GFP contained inside the *Escherichia coli* (top10) through restriction enzyme. 38

4.3 Visualization of *Artemia salina* using fluorescence microscopy 39

CHAPTER 5 DISCUSSION

5.1 Validation of protein 54

5.2 Validation of plasmid 54

5.3 Culture of cysts 55

5.4 Establishment of populations 55

5.5 Feed preparations 56

5.6 Cell washing 56

5.7 Feeding experiments 57

5.8 Visualization of artemia salina 57

CHAPTER 6 CONCLUSION

REFERENCES

APPENDIX

LIST OF TABLES

No.		Page
3.1	Scientific Classification of Artemia Salina	7

LIST OF FIGURES

No. Of Figure		Page
2.1	*Artemia Salina*	6
2.2	Schlösser's drawing of *Artemia* in 1756	9
2.3	The life cycle of brine shrimp	12
2.4	The mating between male and female brine shrimp	13
2.5	Early stages of brine shrimp	14
2.6	Plasmid pGS-21a	18
2.7	Plasmid pDREAM2.1/MCS	19
4.1	Control of the Artemia Salina treated with commercial fish feed pellet with Tetra 40% crude protein	41
4.2	Micrograph of Artemia Salina treated with Escherichia coli BL21 (DE3) Containing the recombinant plasmid PGS-21a-ENTH-GFP.	43
4.3	Micrograph of Artemia Salina treated with Escherichia coli containing the recombinant plasmid pDREAM2.1/MCS-ENTH-GFP	47
4.4	Micrograph of the Artemia Salina that was bombarded with plasmid PGS2.1/MCS-ENTH-GFP	51

LIST OF SYMBOLS

^{o}C	Degree celcius
%	Percentage
µl	Microlitre
µm	Micromithre
L	Litre
M	Molar
cm	Centimetre
mg	Milligram
ml	Millilitre
No.	Number
Rpm	Round per min
Ppt	Part per thousand
s	Seconds
g	Gram
V	Voltan
PBS	Phosphate Buffer Saline
DNA	Deoxyribonucleic Acid

CHAPTER 1

INTRODUCTION

Artemia Salina is classified as a species of Artemia Genus of aquatic crustaceans known as brine shrimp. In the family Artemiidae, Artemia is the only genus that has changed little externally since the Triassic period. *Artemia* is in the order of Anostroca, brings the meaning of 'no shell', which classifies the shrimp with the other species that does not have a hard, bony outer covering. Its subclass Brachiopoda literally means 'gill foot', referring to the fact that the gills are on the outer side of the limb bases. (Banister, 1985; Najarian, 1976).

Artemia Salina is extremely osmotolerant which can be found inhabiting wide variety of water salinity (Van Stappen, 1996, 2002) such as the Great Salt Lake in Northern Utah, on the rocky coast south of San Francisco, the Caspian Sea and other bodies of water with any salt contents, salt swamps near any coast and man-made saltpans around the world (Grizmek, 1972; Pennak, 1989).

Artemia Salina is an arthropod where it has segmented body to which is attached broad leaf-like appendages that greatly increase the apparent size of the animal. For adult male, they can reach total length of 8-10 mm while adult female is 10-12 mm while the width for both sexes is about 4 mm. There are three segments of their body which is head, thorax and abdomen (Criel and MacRae, 2002).

In *Artemia*, two mode of reproduction has been recognized and alternates according to thte conditions of life which are ovoviviparous reproduction (favourable condition) and oviparous reproduction (unfavourable condition) (Lochhead, 1941). In ovoviviparous reproduction, the fertilized egg develops to the stage of gastrula, but instead of being confined, it is differentiate into female body and the larvae called nauplii (free fins larvae). In oviparous reproduction, the fertilized eggs develop into gastrula stage and is surrounded by a tough brown crust consists of protein, lipoprotein, etc. The cysts formed are then released into water. The latter mode of reproduction is predominant at high salinity and lower dissolved oxygen concentrations.

In aquaculture industry, the wide size range of brine shrimp *Artemia Salina* and their different physical forms make them very versatile where the eggs, nauplii and adults of the brine shrimp has been used widely as a life feed for coral, larval fish, and other crustacean. Brine shrimp *Artemia Salina* is important for aquaculture since it is highly nutritious, low cost, and easy to culture (Sorgeloos *et al.*, 1986). In fact, there is still no artificial feed formulation available to completely substitute for *Artemia*.

Besides, its ability to produce dormant eggs, known as cysts, through oviparous reproduction when the environmental conditions are harsh (Criel and MacRae, 2002) can be stored for long periods and hatched on demand has led to extended use of *Artemia* in aquaculture (Daintith, 1996).

Due to its feeding behavior (continuous, nonselective and particle-filter feeder), *Artemia* has been manipulated by many researchers in order to enhance its application in aquaculture life. Indeed, essential nutrients, pigments, prophylatics, and therapeutics may be bioencapsulated in *Artemia* and introduced into consumer organism (Leger *et al.* 1987) and this shows the possibility of using *Artemia* (nauplii or adults) as a carrier for biological molecules components which are otherwise difficult to administer to fish and and crustacean larvae.

Artemia are extensively used as the main food source for the larviculture of marine fish and shrimp where it has successfully been applied as biological carriers for transferring essential nutrients to predator larvae (Leger *et al.*, 1986). Sorgeloos *et al.* (1992), reported on the determination of the accumulation rate of particular drugs in *Artemia* for disease treatment in larviculture.

Bioencapsulation of *Artemia* nauplii for protection against diseases has also been suggested and studied by many groups. Chair *et al.* (1996) reported that antibacterial agents like trimethoprim-sulfamethoxazole can efficiently be encapsulated in *Artemia* nauplii. Accumulation of these therapeutic agents in high quantities occurred when mediated Artemia are fed to larvae of sea bass and post-larvae of white shrimp.

Other than acting as tool for components delivery, *Artemia* larvae also used as a screening system for toxic fungal (Harwig and Scott, 1971) and Brown *et al.* (1968) described a bioassay involving brine shrimp larvae for aflatoxin B_1. *Artemia* has been used widely for quantitation of the potency of anaesthetics (Robinson *et al.*, 1965), evaluation of toxicity of dichloro-diphenyl-trichloroethane (Grosh, 1967) and other insecticides (Michael *et al.*, 1956) of heavy metal salts (Chanh and Mamy, 1963) and antibiotics (Delcambe, 1955).

Objectives of the study

1. To study the capability of *Artemia Salina* to accumulate Green Fluorescent Protein (GFP).
2. To study the capability of *Artemia Salina* to express a GFP gene whose expression is driven by eukaryotic promoter.
3. To investigate the application of the *Artemia Salina* for the delivery of recombinant protein produced in Escherichia Coli using a Green Fluorescent Protein (GFP) reporter as well as a recombinant plasmid PMCS2.1-ENTH-GFP.
4. To study the expression of the PMCS2.1-ENTH-GFP by the *Artemia Salina* after transformation using biolistic gun.

Findings of the study

The findings of this study will facilitate our understanding of the process by which *Artemia* can uptake recombinant proteins and express plasmid DNA constructs containing recombinant protein coding genes. Furthermore, the findings will aids in the enhancement of aquaculture industry.

CHAPTER 2

LITERATURE REVIEW

2.1 The brine shrimp

The brine shrimp *Artemia Salina* is one of the most demanded aquatic animals used and it is organism of economically importance in the aquaculture industry because of its high nutritional value (Sorgeloos *et al.*, 1986). The brine shrimp is outstanding as one of the most researched organism due to its wide distribution all over the world (Hachem *et al.*, 2009) and have more than 500 reported sites (Abatzopoulos *et al.*, 2002).

Brine shrimp filled the food chain between the plankton and larger filter feeders, small fish and birds. According to MacGinite (1968), brine shrimp earn their common name because of their ability to live in a wide range of salinities. Moreover, brine shrimp has the ability which allow them to inhabit an extreme environment of drying salt ponds where little competition occur and able to escape from organism who cannot tolerate high salinity of water (Brown, 1960). It also has fast reproduction cycle and their eggs is able to survive desiccations.

The brine shrimp was first reported in Urmia Lake in 1982 by an Iranian geographer (Asem, 2008). After that, in 1756, Schlösser pictured both sexes of Artemia clearly.

Linnaeus (1758) then described it as *Cancer Salinus* before Leach (1819) transferred it to *Artemia Salina* 61 years later. *Artemia Salina* is a primitive aquatic arthropod with a history for about 100 million years (Asem, 2008).

Figure 2.1 Artemia Salina

2.1.1 Classification

Artemia Salina is classified as a species of Artemia genus of aquatic crustaceans known as brine shrimp. In the family Artemiidae, *Artemia* is the only genus that gas changed little externally since the Triassic period. *Artemia* is in the order Anostroca, literally meaning 'no shell', which classifies the shrimp with either species that have no carapace (a hard, bony outer covering). Its subclass Brachiopoda literally means 'gill foots', referring to the fact that the gills are on the outer side of the limb bases (Banister, 1985; Najarian, 1976). Brachiopoda, from 'branch' meaning gill, and 'poda' meaning feet (Borror, 1988), are characterized by the fact that their appendages are used for locomotion, feeding and respiration.

Table 2.1 Scientific Classification of *Artemia Salina*

SCIENTIFIC CLASSIFICATION	
Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacean
Class	Branchiopoda
Order	Anostraca
Family	Artemiidae
Genus	Artemia
Species	*Artemia Salina* (Linnaeus, 1758)

2.1.2 Natural habitat

Artemia Salina inhabits inland salt lakes such as the Great Salt Lake in Northern Utah, coastal salt lagoons of San Francisco and solar saltworks (Persoone and Sorgeloss, 1980). They also can be found in many salt water bodies with high salinity which varies between 60-300 ppt. Pearse (1987) discovered that they prefer shallow, saline waters and have adapted to the hyper saline waters of drying saltwater ponds.

Artemia is the only Branchiopoda that have adapted to salt water, where this adaptation allows them to escape predation from populations of aquatic animals that cannot tolerate desiccation (Hickman, 1976). *Artemia* are also adapted to widely changeable temperature (6-35°C) and ionic composition, and their pH tolerance varies from neutral to highly alkaline (Van Stappen, 1996, 2002).

It has been shown by Croghan (1957) that *Artemia* has developed a mechanism to maintain haemolymph extreme hypotonic in saline extreme media. This proved that this animal have the ability to reduce the osmotic pressure of haemolyph by NaCl excretion agaisnts the concentration gradient. The brine shrimp also able to overcome the severe physiological demands imposed by these habitats, due to a set of various adaptations, the most salient of which is probably an interchangeable (diapausing cysts versus nauplii) life cycle (Lochhead, 1941).

2.1.3 Characteristics

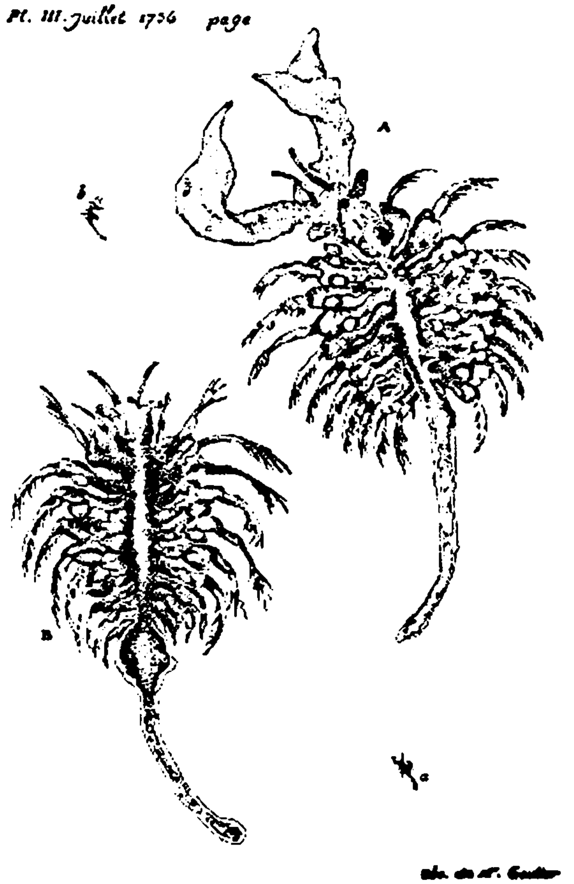

Figure 2.2 Schlösser's drawing of *Artemia* in 1756 (Source: Kuenen & Baas-Becking, 1938)

Artemia Salina's body consists of three segments which are head, thorax, and abdomen though the delineation is often difficult to detect and so the body parts are usually termed as head and trunk (Brown, 1960). A thin exoskeleton is covering the 19 segments of the *Artemia* elongated body. The first 11 of which have pairs of appendages, the next two which are often fused together carrying the reproductive organs and finally the last lead to the tail (Hickman, 1967).

The compound eyes which is located at the end of short stalks helps in detecting the light direction and intensity, though they able to aid in orientation depending on the sunlight's polarization and indicate the present of predators by detecting the light movement (Hickman, 1967).

Their locomotion is reached by the rhythmic beating of the appendages that act in pairs. Telson (a flattened tail) functions as steering and used for rapid movements in order to escape from the predators (Brown, 1960). Respiration occurs on the surface of the legs through fibrous, feather like plates (lamellar epipodites) (Hickman, 1967).

The legs or appendages also aid in the circulation of blood despite the existence of a long tube like heart. The blood which uses haemoglobins as its primary oxygen fixing pigments and lends the brine shrimp its pink colour, does not flow through veins but rather flows in the hemocoelic spaces of the head and throughout the trunk and appendages (Brown, 1960).

They are presenting a sexual dimorphism where the differences of morphological between males and females can be observed in maximum distance between compound eyes, length of first antenna, the width of the third abdominal segment, the total length, diameter of compound eye, length of abdomen.

The adult male can reach up to 8-10 mm long, and the female is 10-12 mm. besides, male brine shrimp have the second antennae markedly enlarged, and modified into clasping organs used in mating differ them from female brine shrimp (Greta, 1980). The other physical differences that can be spotted is the female brine shrimp have brood sac (for eggs storage) and is much larger than the male brine shrimp.

The *Artemia* is a planktivore where it lives on algae, protozoa and detritus. In fact, *Artemia* foraging behavior is an active non-selective filter. Dobbeleir *et al.* (1980) stated that they can ingest small feed particles ranging from 1 to 50 µm in size.

LochHead (1941) reported that the females adult reproduce viviparously or oviparously. These two types of reproduction is alternate depending on the environment condition. Ovoviviparous reproduction occurs when the conditions are favourable where the embryo develops directly into nauplii while oviparous reproduction happens when the conditions are unfavourable, cysts formed.

2.1.4 Life cycle

The life of a brine shrimp started either through ovoviviparous reproduction or oviparous reproduction (Lochhead, 1941). The mating process starts when the male depositing sperm to fertilizes the female's egg by holding the female with his claspers.

REFERENCES

Abatzopoulos, T., J., Kappas, I., Bossier, P., Sorgeloos, P., and Beardmore, J., A. 2002. Genetic characterization of *Artemia tibetiana* (Crustacea:Anostroca). *Biological Journal of the Linnean Society,* **75**: 333-344.

Anderson, D., T. 1967. Larval development and segment formation in the branchipod crustaceans *Limnadea stanleya King* (Conchostraca) and *Artemia salina L.* (Anostraca). *Australian Journal of Zoology,* **15**: 47-91.

Arber, W., Linn, S. 1969. DNA modification and restriction. *Annual Review of Biochemistry.*

Aquila, A., A., Tejeda M., A., Ruiz, M., A. 1994. Using brine shrimp as a drug carrier for therapeutic applications in aquaculture. *Aquacultural Engineering,* **3** (4): 301 -309.

Asem, A. 2008. Historical record on brine shrimp Artemia more than thousand years ago from Urmia Lake, Iran. *Journal of Biological Research-Thessaloniki,* **9**: 113-114.

Banister, K. 1985. *Encyclopedia of Aquatic Life.* New York: Facts on File, Inc.

Borror, Jr., Frank, A. 1988. *Selected Invertebrate Types.* New York, New York: John Wiley & Sons Inc.

Brown, R., F., Wildman, J., D., and Eppley, R., M. 1968. Temperature-dose relationships with atoflaxin on the brine shrimp, *Artemia salina. Journal of (Association of Official Analytical Chemists),* **51**: 905-906.

Brown, Jr., Frank, A. 1960. *Selected Invertebrate Types.* New York, New York: John Wiley & Sons Inc.

Birnboim, H., C., Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research,* **7** (6): 1513–23.

Campbell, Neil, A., and Reece, Jane, B. 2002. *Biology* (6th ed). San Francisco: Addison Wesley. p. 375-401.

Criel, G., and MaacRae, T. 2002. *Artemia* Morphology and Structure. Reproductive Biology of *Artemia.* In: T. Abatzopoulos, J. Beardmore, J. Clegg, P. Sorgeloos (Eds.). *Artemia: Basic and Applied Biology.* Kluwer Academic Publishers.

Cremer, C., Hell, S., W., Stelzer E., H., K., Lindek, S., Stelzer, Lindek. (1994). Confocal microscopy with an increased detection aperture: type-B 4Pi confocal microscopy. *Optics Letters*, **19** (3):222–224.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W., W., Prasher, D., C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, **263**: 802-805.

Chair, M., Nelis, H., J., Leger, P., Sorgeloos, P., Deleenheer, A., P. 1996. Accumulation of rimethoprim, sulfamethoxazole, and N-acetylsulfamethoxazole in fish and shrimp fed medicated *Artemia franciscana*. *Antimicrobial Agents Chemotherapy*, **40**: 1649- 1652.

Chang, S., H., Lee, B., C., Chen, Y., D., Lee, Y., C., and Tsai, H., J. 2011. Development of transgenic zooplankton *Artemia* as a bioreactor to produce exogenous protein. *Transgenic research*, **20** (5): 1099-1111.

Chanh, P., H., and Many, G. 1963. A simple biological reagent for toxicity tests: The eggs of *Artemia* species by Fusarium solani. *Bulletin-European Association of Fish Pathologists*, **9**: 35-37. Delcambe, L. 1955. Activities biologiques des Actinomycetes de Streptomycetes S-67. *Archive International Pharmacodyn Therapy*, **101**: 358-366.

Croghan, C. 1957. The osmotic and ionic regulation of *Artemia salina* (L.). Department of Zoology, University of Cambridge.

Criado-Fornelio, A., Mialhe, E., Constantin, E., and Grizel, H. 1989. Experimental infection of *Artemia* species by *Fusarium solani*. *Bulletin-Europe Association Fish Pathologist*, **9**:35- 37.

Daintith, M. 1996.Rotifers and *Artemia* for Marine Aquavulture: *A Training Guide*. Tasmania: University of Tasmania.

Dobbeleir, J., Adam, N., Bossuyt, E., Buggeman, E., and Sorgeloos, P. 1980. Newaspects of the use of inert diets for high density culturing of brine shrimp. In: Persoone, G., Sorgeloos, P., Roels, O., Jasper, E. (Eds.). *The brine shrimp Artemia ecology, culture, use in aquaculture*. Vol. 3. Wetteren, Belgium: Universal Press. p. 165 -174.

Thomas, M., Christopher and Summers, David. 2008. Bacterial Plasmids. In: *eLS*. John Wiley & Sons inc.

Escalante, R., and Sastre, L. 1994. Structure of *Artemia franciscana* sarco/endoplasmic reticulum Ca-ATPase gene. *Journal of Biological Chemistry*, **269**: 3005-3012.

Granvil, D.,T. 2000. *Artemia* production for marine larval fish culture. Southern Regional Aquaculture Centre. *Aquaculture* **155**: 135-148

Grzimek, B. 1972. *Grzimek's Animal Life Encyclopedia, Vol. 1 "Lower Animals".* New York: Van Nostrand Reinhold Co.

Gendreau, S., Lardans, V., Cadoret, J., P., and Mialhe, E. 1995. Transient expression of a luciferase reporter gene after ballistic introduction into *Artemia franciscana* (Crustacea) embryos. *Aquaculture,* **133** (3): 199-205.

Gatesoupe, F. 1994.lactic acid bacteria increase the resistance of turbot larvae (*Scopthalmus maximus*) against pathogenic *Vibrio. Aquatic Living reservoir,* **7**: 277-282.

Greta, E., T., and Michael, L., S. 1980. Scanning electron microscopy of the frontal knobs of the male brine shrimp. *Transactions of the American Microscopical Science,* **99** (2): 167- 172.

Gan, C. 1989. Gene gun accelerates DNA-coated particles to transform intact cells. *The Scientist,* **3** (18): 25.

Grosh, D., S. 1967. Poisoning with DDT: Effect on reproductive performance of *Artemia. Science,* **155**: 592-593.

Hachem, B., N., Amal, B.,R., J., Mohamed, S., R. 2009. New distribution record of the brine shrimp Artemia (Crustacea, Branchiopoda, Anostroca) in Tunisia. *Check List* **5**(2): 281–288, 2009.

Hickman, and Cleveland, P. 1967. *Biology of the Invertebrates.* St. Louis, Missouri: C.V. Mosby Company.

Harwig, J., and Scott, P., M. 1971. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxics. *Applied microbiology,* **21** (6), 1011-1016.

Kuenen, D., J., and Baas-Becking, L., G., M. 1983. Historical notes on Artemia salina. *Zoologischer Mededeelingen,* **20**: 222-230.

Kessler, C. 1990. Detection of nucleic acids by enzyme-linked immune-sorbent assay (ELISA) technique: An example for the development of a novel non-radioactive labeling and detection system with high sensitivity. In: *Advances in Mutagenesis Research.* Springer-Verlag, Berlin. pp. 105-152.

Leger, Ph., Bengston, D., A., Simpson, K., L., and Sorgeloos, P., 1986. The use and nutritional value of *Artemia* as a food source. P. 521-623. In: *Oceanography and*

Marine Biology. Annual Revision., Vol. 24. Barnes, M., (Ed.). Scotland: Aberdeen University Press. p.687.

Leger, Ph., Naessens-Foucquaert, E., and Sorgeloos, P., 1987. International Study on *Artemia*, XXXIV. Techniques to manipulate the fatty-acid profile in *Artemia* nauplii and the effect on its nutritional effectiveness for the marine crustacean *Mysidopsis bahia* (M.). In: *Artemia Research and its applications.* Vol. 3. Sorgeloos, P., Bgston, D., A., Decleir, W., and Jespers, E., (Eds.). Belgium: Universal Press, Wetteren.

Lochhead, J. H., 1941. Artemia, the Brine Shrimp. *Turtox News*, **19**: 41-45

Lederberg, J. 1952. Cell genetics and hereditary symbiosis. *Physical Review,* **32** (4): 403–430.

MacGinite, G., E., and Nettie MacGinite. 1968. *Natural History of mairne animals 2nd Ed.* New York, New York: McGraw-Hill Financial Inc.

Maria T., M., and Leandro Sastre (1990). Molecular cloning and expression of four actin isoforms during *Artemia* development. Journal of Nucleic Acids Research, **18** (17): 5219-5225.

Marques, A., Francois, J., Dhont, J., Bossier, P., and Sorgeloos, P. 2004. Influence of yeast quality on performance of gnotobiotically-grown *Artemia. Journal of Experimental Marine Biology and Ecology*, **310**: 247-264.

Michael, A., S., and Bland, C. 1981. Infection of *Artemia salina* by *Haliphtoros milfordensis*: ascanning and transmission electron microscope study. *Journal Invertebrate Pathology*, **37**: 249-257.

Najarian, H. 1976. *Sex Lives of Animals without Backbones.* New York: Charles Scribner's Sons.

Ormö, M., Cubitt, AB., Kallio, K., Gross, L., A., Tsien, R., Y., Remington, S., J. (September 1996). Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science,* **273** (5280): 1392–5.

Overton, S., and Bland, C. 1981. Infection of *Artemia salina* by *Haliphtoros milfordensis*: a scanning and transmission electron microscope study. *Journal Invertebrates Pathology*, **37**: 249-257.

Pennak, R. 1989. *Fresh-Water Invertebrates of the United States*. Canada: John Wiley and Sons, Inc.

Phillips, G. 2001. "Green fluorescent protein--a bright idea for the study of bacterial protein localization". *Federation of European Microbiological Societies (FEMS) Microbiology Letters*, **204** (1): 9–18.

Persoone, G., and Sorgeloos, P. 1980. General aspects of the ecology and biogeography of Artemia; p. 3-24. In: Persoone, G., Soegeloos, P., Roels, O., and Jaspers, E. (Eds.). *The brine shrimp Artemia. Vol. 3. Ecology culturing use in aquaculture.* Belgium/Osost laanderen: University Press, Wettern.

Pearse, Viki, et al. 1987. *Living Invertebrates.* Palo Alto, California: Blackwell Scientific Publications.

Robinson, A., B., Manley, K., F., Anthony, M., P., Catchpool, J., F., and Pauling, L. 1965. Anesthesia of Artemia larvae: Method for quantitative study. *Science*, **149**: 1255 -1258.

Roberts, R.,J. 1976. "Restriction endonucleases". *CRC Critical Review in Biochemistry*, **4** (2): 123–164.

Rosa, G., C., Sergio, G.,R., and Carlos G., V. 1998. The post- embryonic development of Artemia persimilis piccineli & Prosdocimi. *Hydrobiology.* **391**: 63-80

Rath, Arianna and Glibowicka, Mira and Nadeau, Vincent G. and Chen, Gong and Deber, Charles, M. 2009. "Detergent binding explains anomalous SDS-PAGE migration of membrane proteins". *Proceedings of the National Academy of Sciences*, **106** (6): 1760-1765.

Rengpipat, S., Phianpak, W., Piyatiratiticorakul, S., and Menasveta, P., 1998. Effect of probiotics on Black tiger shrimp *Penaues monodon* survival and growth. *Aquaculture.*, **167**: 301-313.

Subharda, B., Hurwitz, I., Fieck, A., Rao, D., V., S., Subba R., G., and Durvasula, R. 2010. Development of paratransgenic *Artemia* as a platform for control of infectious diseases in shrimp mariculture. *Journal of Applied Microbiology*. **108** (3): 831-840.

Supattra Treerattrakool, Chuthakorn Chartthai, Nipatthra Phrommain, Sakol Panyim, and Apinunt Udomkit. Silencing of gonad- inhibiting hormone gene expression in *Panaeus monodon* by feeding with GIH dsRNA-enriched. *Artemia aquaculture*, **404-405**: 116-121.

Sambrook, J., Peter MacCallum. 2000. *Molecular Cloning: A Laboratory Manual.* Third Edition. Melbourne, Australia: Cancer Institute. Cold Spring Harbor, New York.

Schrehardt, A. 1987. A scanning electron-microscopic study of the post-embryonic development of Artemia. In: *Artemia Research and Applications.* **1**:5-32. Wetteren, Belgium: Universal Press.

Sorgeloos, P., and Lavens, P. 1987. The cryptiobiotic state of Artemia cysts, its diapause deactivation and hatching: a review. In: *Artemia research and its applications.* Vol. 3. Sorgeloos, P., Bengston, D., A., Decleir, W., and Jaspers, E. (Eds.) Universal Press, Wetteren, Belgium.

Sorgeloos, P., Lavens, P., Leger, P., Tackaert, W., and Versichele, D. 1986. *Manual for the Culture and Use of Brine Shrimp in Aquaculture.* Belgium: Artemia Reference Center,Faculty of Agriculture, State University of Ghent: 1986: 91-95.

Sorgeloos, P., Verpraet, R., Chair, M., Leger, Ph., Nelis, J., P., and De Lenheer, A., P. 1992. Live food mediated drug delivery as a tool for disease treatment in larviculture. The enrichments of therapeutics in rotifers and Artemia nauplii. *Aquacultural Engineering,* **11**: 133-139.

Tida Pechmanee. 1989. Report on the training course on Sea-bass breeding and culture. Fish and aquaculture department, P87. Wallingford, UK. pp. 40–50.

Tsien, R., Y. 1998. The green fluorescent protein. *Annual Review Biochemistry,* **68**: 509 -44

Van Stappen, G. 1996. Introduction, Biology and Ecology of Artemia. In: P. Lavens, P. Sorgeloos (Eds.). *Manual on the Production and Use of Live Food for Aquaculture.* FAO Fisheries Technical paper 361.

Van Stappen, G. 2002. Zeegeography. In: T. Abatzopoulos, J. Beardmore, J. Clegg, P. Sorgeloos (Eds.). *Artemia: Basic and Applied Biology.* Kluwer Academic Publishers.

Verchuere, L., Rombaut, G., Sorgeloos, P., and Verstraete, W. 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews,* **64** (4): 655-671.

Vershuere, L., Heang, H., Sorgeloos, P., and Verstraete, W. 2000. Selected bacteria strains protect Artemia species from pathogenic effects of Vibrio proteolyticus. *Applied Environmental Microbiology,* **66** (3): 1139-1146.

Vershuere, L., Romabut, G., Huys, G., Dhont, J., Sorgeloos, P., and Verstraere, W. 1999. Microbial control of the culture of *Artemia* juveniles through pre-emptive colonization by selected bacterial strains. *Applied Environmental Microbiology,* **65** (6): 2527-533.

Watson, James, D. 2007. *Recombinant DNA: Genes and Genomes: A short Course.*

Watanebe, T., Kitajama, C., and Fujita, S. 1983. Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture*, **34**: 115-143.