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
UNIVERSITI MALAYSIA SABAH

Borang Pengesahan Status Tesis

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

Ijazah: IJAZAH SARJANA MUDA BIOTEKNOLOGI

Saya: NUR DAYU BINTI ADNAN (HURUF BESAR)

(Sesi Pengajian: 2011/2014)

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

1. Tesis adalah hak milik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuka salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuka salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/):
   - SULIT
     (Mengandungi maklumat yang berdasarkan kepentingan nasional atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)
   - TERHAD
     (Mengandungi maklumat TERHAD yang telah disentuh oleh organisasi/badan di mana penyelidikan dijalankan)
   - 1.DAK TERHAD

Daftar oleh

NURULAIN BINTI ISMAIL
LIBRARIAN
UNIVERSITI MALAYSIA SABAH

NAMA PENYELIA

Dr Kenneth Rodrigues

Tarikh: 26 Jun 2014

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

PERPUSTAKAAN UMS

*1000358157*
DECLARATION

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

NUR IDAYU BINTI ADNAN
(BS 11110480)
26 JUNE 2014
VERIFICATION

SUPERVISOR
( DR KENNETH F. RODRIGUES )

SIGNATURE

[Signature]
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 Shaffiy 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

CHAPTER 1 INTRODUCTION

CHAPTER 2 LITERATURE REVIEW

2.1 *The Brine Shrimp*  
2.1.1 Classification  
2.1.2 Natural Habitat  
2.1.3 Characteristics  
2.1.4 Life Cycle

2.2 Application  
2.2.1 Application in Aquaculture  
2.2.2 Other Application

2.3 ENTH-GFP

2.4 Plasmid  
2.4.1 pGS-21a  
2.4.2 pDREAM2.1/MCS
2.5 Bacterial Strains
   2.5.1 *Escherichia coli* BL21 (DE3)
   2.5.2 *Escherichia coli* (TOP10)

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

---

**CHAPTER 3 METHODOLOGY**

3.1 Validation of plasmid DNA by using restriction enzyme method
   3.1.1 Plasmid extraction
   3.1.2 DNA and plasmid quantification
   3.1.3 Restriction enzyme
   3.1.4 Gel electrophoresis

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

3.3 Culture of cysts

3.4 Establishment of populations

3.5 Preparations for feeds (protein and plasmid purposes)
   3.5.1 Culture of plasmid pGS21-a-ENTH-GFP in *Escherichia coli* BL21 (DE3) cells
   3.5.2 Culture of plasmid pMCS2.1-ENTH-GFP in *Escherichia coli* (TOP10) cells
### Chapter 3.6 Cell washing

### Chapter 3.7 Feeding experiment

### Chapter 3.8 Sampling for visualization

### Chapter 3.9 Visualization using fluorescence microscopy
- **3.9.1** Microscope slide preparation
- **3.9.2** Visualization under fluorescence microscope

### Chapter 3.10 Transformation of cysts using biolistic gun
- **3.10.1** Plasmid extraction and purification
- **3.10.2** Sample (cysts) preparation
- **3.10.3** Coating washed microcarriers with DNA
- **3.10.4** Preparation for bombardment
- **3.10.5** System bombardment set up
- **3.10.6** Culture and visualization of the bombarded cysts

### 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.

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

**4.3** Visualization of *Artemia salina* using fluorescence microscopy

### Chapter 5 DISCUSSION

**5.1** Validation of protein

**5.2** Validation of plasmid
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3 Culture of cysts</td>
<td>55</td>
</tr>
<tr>
<td>5.4 Establishment of populations</td>
<td>55</td>
</tr>
<tr>
<td>5.5 Feed preparations</td>
<td>56</td>
</tr>
<tr>
<td>5.6 Cell washing</td>
<td>56</td>
</tr>
<tr>
<td>5.7 Feeding experiments</td>
<td>57</td>
</tr>
<tr>
<td>5.8 Visualization of artemia salina</td>
<td>57</td>
</tr>
</tbody>
</table>

CHAPTER 6 CONCLUSION

REFERENCES

APPENDIX
# LIST OF TABLES

<table>
<thead>
<tr>
<th>No.</th>
<th>Table Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Scientific Classification of Artemia Salina</td>
<td>7</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

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

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degree celcius</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micromithre</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>No.</td>
<td>Number</td>
</tr>
<tr>
<td>Rpm</td>
<td>Round per min</td>
</tr>
<tr>
<td>Ppt</td>
<td>Part per thousand</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>V</td>
<td>Voltan</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
</tbody>
</table>
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 salt pans 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 modes of reproduction have been recognized and alternate according to the conditions of life which are ovoviviparous reproduction (favorable condition) and viviparous reproduction (unfavorable condition) (Lochhead, 1941). In ovoviviparous reproduction, the fertilized egg develops to the stage of gastrula, but instead of being confined, it is differentiated into female body and the larvae called nauplii (free fins larvae). In oviparous reproduction, the fertilized eggs develop into gastrula stage and are surrounded by a tough brown crust consisting 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, prophylactics, 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 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 B₁. 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).

![Artemia Salina](image)

**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 has 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 feet', 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*

<table>
<thead>
<tr>
<th>SCIENTIFIC CLASSIFICATION</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td>Animalia</td>
</tr>
<tr>
<td>Phylum</td>
<td>Arthropoda</td>
</tr>
<tr>
<td>Subphylum</td>
<td>Crustacean</td>
</tr>
<tr>
<td>Class</td>
<td>Branchiopoda</td>
</tr>
<tr>
<td>Order</td>
<td>Anostraca</td>
</tr>
<tr>
<td>Family</td>
<td>Artemiidae</td>
</tr>
<tr>
<td>Genus</td>
<td>Artemia</td>
</tr>
<tr>
<td>Species</td>
<td><em>Artemia Salina</em> (Linnaeus, 1758)</td>
</tr>
</tbody>
</table>

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 haemolymph by NaCl excretion againsts 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 haemoglobin 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 claspers 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


