Genetic and Biochemical evaluation of Brine shrimp, *Artemia* from the hypersaline habitats of Indian subcontinent

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VIKAS P.A
(Reg. No. MU/EXB/Ph.D/Bio.Sc/CR.03/2008-09/E.13, dated 02.12.2008)

Under the Faculty of Science and Technology

MANGALORE UNIVERSITY
Mangalagangotri - 574 199
Dakshina Kannada Dist. Karnataka, India

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
Post Box No. 1603, Cochin-682 018, Kerala, India

March 2013

Dedicated to the Aquaculture development initiatives in India…..

Declaration

I do hereby declare that the thesis entitled "**Genetic and Biochemical evaluation of Brine shrimp, *Artemia* from the hypersaline habitats of Indian subcontinent**" is an authentic record of research work carried out by me under the guidance and supervision of Dr. P. C. Thomas, Principal Scientist & SIC HRD, Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Kochi in partial fulfilment for the award of Ph.D degree under the Faculty of Biosciences of Mangalore University and no part thereof has been previously formed the basis for the award of any diploma or degree, in any University.

Cochin **(Vikas P.A)**
10/03/2013

Phone : (Off) : 2394867/.........Ext.
2391407
Telegram : CADALMIN, EKM
Telex : 0885-6435 MFRI IN
Fax : 0091-0484- 2394909/2396685
E-mail : mdcmfri@md2.vsnl.net.in

केंद्रीय समुद्री मात्स्यिकी अनुसंधान संस्थान
कोच्ची - 682 018, केरल, भारत
भाकृ अनुप
ICAR
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
(Indian Council of Agricultural Research)
P.B No. 1603, Ekm North PO,COCHIN 682018, Kerala, India

Dr. P. C. Thomas
Principal Scientist & SIC HRD Cell
Marine Biotechnology Division
(Supervising Guide)

Date: 10/03/2013

Certificate

*This is to certify that this thesis entitled "***Genetic and Biochemical evaluation of Brine shrimp, Artemia from the hypersaline habitats of Indian subcontinent** *" is an authentic record of research work carried out by* **Mr. P.A Vikas**, *under my guidance and supervision in Central Marine Fisheries Research Institute, Cochin, in partial fulfilment of the requirement for the award of the degree of* **Doctor of Philosophy** *in the* **Faculty of Biosciences in Mangalore University**. *The thesis or part thereof has not previously been presented for the award of any Degree in any University.*

(P. C. Thomas)

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Preface

Aquaculture is one of the fastest growing animal food producing sectors in the world, accounting for almost half of the total food fish supply. Sustainable larval production is vital to the success of aquaculture. Non availability of healthy, uniform sized, quality seeds in sufficient quantities have always been a concern in ‘capture based aquaculture’ where seeds are sourced from the wild. This issue could be addressed through the hatchery production of seeds that can ensure the availability of quality seeds in required quantities. The use of hatchery developed seeds may also relieve the pressure on the wild caught seeds. All these have forced the Aquaindustry to move towards ‘hatchery based aquaculture’ systems. In hatcheries, live feeds play a key role in deciding the survival, growth and development of the larvae. Due to the high growth rate in the early developmental phase of the larvae, the starter diet should hold high levels of essential nutrients. Most of the finfish larvae are small with narrow buccal openings, and therefore, exogenous foods of small size are needed one or two days after hatching. Therefore, the success of a starter diet in larviculture lies in its size and its nutrient content because the feed has to be perceived, captured, accepted and efficiently ingested by the larvae. Among the different live feeds, *Artemia* are widely used as the starter diet in larviculture. Its storage ability, easiness to hatch on demand and their soft texture make them quite essential in marine larviculture. Different *Artemia* sp. are widely distributed on the five continents in many salt lakes, coastal lagoons and solar salt works. To lessen the dependency on natural *Artemia* sources and reduce the larval rearing expenses, various formulated diets have been tried as alternatives. But, due to reduced preference/acceptability by the larvae, water quality deterioration, etc. their acceptance got reduced in larviculture. Although, all stages of *Artemia* are suitable diet for diversified group of finfish and shellfish larvae, nauplii stage is considered as the most preferred larval diet. Hatching efficiency, nauplii size and nutrient profile are the foremost indicators that determine the quality of the *Artemia* of which nauplii size is the most important one in larviculture. In India, presence of *parthenogenetic Artemia* was first reported by different researchers since 1953. However, these strains have never been exploited. Instead during the last three decades the Indian aquaculture industry have been importing exotic *Artemia* strains. It is suspected that the large scale import of exotic *Artemia* have resulted in the invasion of the Indian waters by them. The chance for the extinction of native species is more when the congeneric native species exist in the same niche/ habitat. Therefore, detailed survey for the collection and identification of the indigenous *Artemia* is essential to verify the suspicion. Though, different *Artemia* strains are found widely distributed in various hypersaline habitats across the globe, based on their nutrient quality, small naupliar size and hatching efficiency, only few strains are used extensively as starter diet in larviculture. Though, *Artemia* strains with higher nutrient qualities are preferred and have great demand in aquaculture, very few attempts have been made to evaluate the nutrient quality and suitability of indigenous *Artemia* strains as live feeds for Indian aquaculture. It is essential to evaluate the nutritional content along with the life history characters of indigenous *Artemia* strains to determine its suitability. A key concern in the larval rearing process is the timely availability of small size nauplii. Various approaches are being adopted to selectively harvest small size live prey to be used as starter diet for small mouthed larvae. Genetic manipulation is an ideal technique to bring about desired change in nauplii size like any other biological trait. Selective breeding is a time tested quantitative genetic manipulation technique employed in plants and livestock, and can be used to develop *Artemia* producing small nauplii. *Artemia* nauplii lack certain essential nutrients required for the marine fish larvae. Hence, it is necessary to meet the essential PUFA requirement, through the enrichment of the above live feeds. Commercial enrichment diets are widely used for this purpose. As an alternative to the commercial enrichment diets, there is a growing interest in the aqua-industry to use marine microalgae as enrichment diet for the live feeds. In this context, a detailed study on pre and post enrichment effects of microalgae diet on the *Artemia* nauplii is valuable to develop an enrichment protocol and assess their superiority over commercial enrichment formulations. *Artemia* are rarely found in waters with salinity lower than 45 ppt, although physiologically they thrive in seawater and even in brackish waters. Salinity is without any doubt the predominant abiotic factor determining the presence of *Artemia* and the natural defense mechanism against predators consequently limiting its geographical distribution. During stress, molecular chaperones interact with other proteins to modulate folding, cell localization and functionality, and to protect against irreversible denaturation, and the small heat shock proteins are noted for their protective capability, representing the first line of defense against physiological and environmental stresses. Hence the effect of salinity stress on sHSP expression and biochemical constituents such as trehalose, carotenoids, fatty acids, amino acids, protein etc., was studied.

Abbreviations

%	: Percentage
<	: Greater than
>	: Smaller than
µ	: Overall mean
µl	: Micro liter
µm	: Micro meter
µM	: Micro molar
µmol	: Micro mole
π	: Nucleotide diversity
σ^2p	: Sire component of variance
σp	: Phenotypic standard deviation
σ^2_W	: Error variance component
^0C	: Degree Celsius
18:3*n*-3	: Alpha-linolenic acid
20:4n 6	: Arachidonic acid
20:5*n*-3	: Eicosapentaenoic acid
22:6*n*-3	: Docosahexaenoic acid
AA	: Amino acid
Ala	: Alanine
ANFH	: Average nauplii at first hatch
ANOVA	: Analysis of Variance
Arg	: Arginine
ArHsp21	: *Artemia* heat shock protein 21
ArHsp22	: *Artemia* heat shock protein 22
ASL	: *Artemia salina*
Asp	: Aspartic acid
AUFS	: Absorbance units full scale
BF_3	: Boron trifluride
BLAST	: Basic Local Alignment Search Tool
Bp	: Base pairs
BSA	: Bovine serum albumin
CD	: Cyst diameter
$CHCl_3$-CH_3OH	: Chloroform methanol
CMFRI	: Central Marine Fisheries Research Institute
$CoCl_2$	: Cobalt chloride
CO1	: Cytochrome c oxidase subunit 1
$CuSO_4$	: Copper Sulphate
Cys	: Cystine
DAA	: Dispensable Amino Acid

DF	: The degree of freedom
DHA	: Docosahexaenoic acid
DNA	: Deoxyribo Nucleic Acid
dNTPs	: Deoxynucleoside tri phosphates
EDTA	: Ethylene Diamine Tetra Acetic acid
EPA	: Eicosapentaenoic acid
F2	: Generation two
FAME	: Fatty acid methyl ester
FAO	: Food and Agriculture Organization
FCR	: Feed conversion ratio
FDL	: First day length
$FeCl_3$	: Ferric chloride
GC	: Guanine-cytosine
GLC	: Gas-liquid chromatography
Glu	: Glutamine
Gly	: Glycine
gm	: Gram
GMJ	: Mithapur
GNM	: Nanganvadi
GSL	: Great salt lake
H	: Height
h^2	: Heritability
H_2O	: Water
H_3BO_3	: Boric acid
HCl	: Hydro chloric acid
His	: Histidine
HP	: Hatching percentage
HPLC	: High performance liquid chromatography
IAA	: Indispensable Amino Acid
Ile	: Isoleucine
IPCC	: Intergovernmental Panel on Climate Change
ITS-1	: Internal transcribed spacer 1
K_1	: Average number of progeny per sire
KCl	: Potassium chloride
KNO_3	: Potassium nitrate
KOH/MeOH	: Potassium hydroxide solution in methanol
L	: Length
Leu	: Leucine
LFS	: Length of female at sexual maturity
LFSM	: Length of female at sexual maturity
LMS	: Length of male at sexual maturity

LMSM	: Length of male at sexual maturity
Lux	: Lux
Lys	: Lysine
M	: Meter
Me3N	: Trimethylamine
Met	: Methionine
mg	: milligram
MgCl	: Magnesium chloride
Min	: Minute
ml	: Millilitre
mM	: Millimolar
mm	: Milli meter
$MnCl_2$	: Manganese(II) chloride
MP	: Maximum parsimony
MS	: Mean sum of squares
MUFA	: Monounsaturated fatty acids
Na_2HPO_4	: Disodium hydrogen phosphate
NaCl	: Sodium chloride
NaHPO4	: Sodium phosphate
NaOH	: Sodium hydroxide
NCBI	: National Centre for Biotechnology Information
ND	: Not Detected
nDNA	: Nuclear DNA
ng	: Nanogram
$NH_46MO_7O_{24}$	: Ammonium heptamolybdate
NJ	: Neighbor joining
NL	: Nauplii length
nm	: Nanometer
OD	: Optical density
PCoA	: Principal Coordinate Analysis
PCR	: Polymerase Chain Reaction
Phe	: Phenylalanine
PITC	: Phenylisothiocyanate
ppt	: Parts per thousand
Pro	: Proline
PTC	: Phenylthiocabamyl
PUFA	: Polyunsaturatted Fatty Acids
R	: Average predicted response per generation
rDNA	: ribosomal DNA
RNA	: Ribonucleic acid
rpm	: Rotations per minute

SBAS	: Selectively bred *Artemia* strain
SD	: Standard deviation
SDL	: Sixth day length
SDS	: Sodium Dodecyl Sulphate
SE	: Standard Error
Ser	: Serine
SFA	: Saturated fatty acids
SFB	: San Francisco bay Strain
SGR	: Specific growth rate
sp.	: Species
SS	: Sum of squares
TBS	: *Artemia tibetiana*
TDL	: Third day length
TFA	: Total fatty acid
Thr	: Threonine
TL	: Total length
TLC	: Thin layer chromatography
TNM	: Thamaraikkulam
TT	: Transitions to transversions
TTJ	: Tuticorin
Tyr	: Tyrosine
USA	: United States of America
UV	: Ultraviolet
v/v	: Volume/volume
Val	: Valine
VDA	: Vedaranyam
VLCFA	: Very long-chain fatty acids
VVC	: Vietnam
$ZnCl_2$	: Zinc chloride

Contents

List of Abbreviations
List of Tables
List of Figures

1. General Introduction: 1-10

2. Molecular and morphologic characterization of Brine Shrimp, *Artemia sp.* 11-51

2.1. Abstract 11

2.2. Introduction and review of literature 13

2.3. Materials and methods 18

2.3.1. Hatching of cyst 21

2.3.2. Microalgae culture for Artemia rearing 21

2.3.3. Evaluation of cyst hatching percentage 22

2.3.4. Morphological characterization 23

2.3.5. Reproductive pattern 24

2.3.6. Crossbreeding experiments 24

2.3.7. Molecular genetic characterization 26

2.3.7.1. DNA isolation 26

2.3.7.2. Amplification and sequencing of internally transcribed spacer region-1 (ITS-1) 27

2.3.7.3. Sequencing and Phylogenetic analysis: 28

2. 4. Results 29

2.4.1. Hatching percentage 30

2.4.2. Morphological observations 30

2.4.3. Cyst biometry 32

2.4.4. Reproductive pattern 33

2.4.5. Molecular genetic characterization 35

2.5. Discussion 41

2.6. Conclusion 50

3. Evaluation of nutrient composition of Artemia strains collected from Indian salinas............53-90

3.1. Abstract 53

3.2. Introduction and review of literature 55

3.3. Materials and methods............ 59

3.3.1. Biochemical analyses of different stages of Artemia 61

3.3.1.1. Chemicals and instrumentation............ 61

3.3.1.2. Estimation of soluble protein 61

3.3.1.3. Estimation of total carotenoids............ 62

3.3.1.4. Estimation of amino acids............ 62

3.3.1.5. Estimation of trehalose 65

3.3.1.6. Estimation of fatty acids............ 65

3.3.2. Statistical analyses 66

3.4. Results............ 66

3.4.1. Nutrient evaluation............ 66

3.4.1.1. Soluble protein content............ 66

3.4.1.2. Carotenoid content............ 67

3.4.1.3. Trehalose content............ 67

3.4.1.4. Amino acid content............ 70

3.4.1.5. Fatty acid composition 76

3.5. Discussion 81

3.6. Conclusions............ 90

4. Selective breeding for reduction of nauplii size in the indigenous Artemia strain 91-111

4.1. Abstract 91

4.2. Introduction............ 92

4.3. Materials and methods............ 95

4.3.1. Collection of Artemia cysts 95

4.3.2. Hatching and rearing............ 95

4.3.3. Selective Breeding 95

4.3.4. Recording and analysis of nauplii length, cyst biometry and hatching percentage 96

4.3.5. Heritability estimation 97

4.3.6. Selection differentials 100

4.3.7. Statistical analysis 101

4.4. Results 101

4.4.1. Phenotypic parameters 101

4.4.2. Heritability estimates of nauplii length 102

4.4.2.1. Selection differentials 103

4.4.2.2. Response to selection 104

4.4.3. Correlated response 105

4.5. Discussion 107

4.5.1. Heritability of the selected trait 109

4.6. Conclusion 111

5. Biochemical and biometrical evaluation of Artemia nauplii enriched with Microalgae 113-137

5.1. Abstract 113

5.2. Introduction 114

5.3. Materials and methods: 117

5.3.1. Preparation of microalgae culture for enrichment 117

5.3.2 Cyst hatching and enrichment of Artemia nauplii 118

5.3.3. Evaluation of growth and survival rates of the enriched nauplii 118

5.3.4. Evaluation of salinity stress tolerance of the enriched nauplii 119

5.3.5. Estimation of fatty acid content of the enriched nauplii 119

5.4. Results 120

5.4.1. Effect of enrichment time on the total length of the nauplii 120

5.4.2. Mortality percentage of Artemia nauplii during enrichment........ 125

5.4.3. Salinity tolerance in enriched Artemia nauplii 125

5.4.4. Effect of enrichment time on the fatty acid profile of the nauplii.. 127

5.5. Discussion 133

5.6. Conclusions.. 137

6. Differential expression of biochemical constituents and real time analysis of ArHsp22 gene in Artemia under salt stress..............................139-175

6.1. Abstract 139

6.2. Introduction and review of literature 141

6.3. Materials and methods.. 147

6.3.1. Experimental animals.. 147

6.3.2. Experimental setup.. 147

6.3.2.1. Salinity stress treatment...147

6.3.3. Evaluation of survival rate 148

6.3.4. Sampling for biochemical and molecular analysis........................ 148

6.3.5. Biochemical analysis.. 149

6.3.5.1. Estimation of soluble protein..149

6.3.5.2. Estimation of total carotenoids...149

6.3.5.3. Estimation of amino acids...150

6.3.5.4. Estimation of trehalose 150

6.3.5.5. Estimation of fatty acids..151

6.3.6. Molecular analysis... 151

6.3.6.1. RNA isolation and cDNA synthesis..151

6.3.6.2. Real time–PCR and quantitative analysis...............................152

6.3.7. Statistical analyses 155

6.4. Results.. 155

6.4.1. Mortality percentage of Artemia subjected to salt stress............. 155

6.4.2. Variation in soluble protein content in Artemia under salt stress.. 156

6.4.3. Changes in total carotenoid content in Artemia under salt stress.. 156

6.4.4. Variation in trehalose content in Artemia under salt stress......... 157

6.4.5. Variation in fatty acid content in Artemia under salt stress........ 157

6.4.6. Variation in amino acid content in Artemia under salt stress...... 160

6.4.7. ArHsp 22 gene expression in Artemia under salt stress................ 165

6.5. Discussion.. 167

6.6. Conclusion... 175

7. Summary and Conclusion..177-193

8. Reference...195-221

9. Publications 223-262

10. Appendix..263-269

List of Tables

Table No.	Title	Page No.
Table 2.1	Collection location, date of collection, Geographical Coordinates, Nature of Sample and Gender of the Artemia samples collected from Indian Salinas	20
Table 2.2	Mean values of life history traits of the geographical strains of indigenous Artemia	32
Table 2.3	Life history traits of the F1 generation cross bred Artemia	34
Table 2.4	Sample ID, Collection site and Gen Bank Accession number of ITS-1 sequence submissions from the present study	36
Table 2.5	Haplotype diversity of the different Artemia strains	37
Table 2.6	Sequence characteristics of DNA segment comprising of Partial 18S rRNA, complete ITS1 partial 5.8S rRNA	38
Table 2.7	Mean genetic distances of indigenous Artemia from other Artemia species.	38
Table 3.1	HPLC gradient elution programme for the determination of amino acids	64
Table 3.2	Protein, carotenoid and trehalose content of life cycle stages of indigenous Artemia strains.	69
Table 3.3	Amino acid content of the Artemia cyst	71
Table 3.4	Amino acid content of the Artemia nauplii	73
Table 3.5	Amino acid content of the adult Artemia	75
Table 3.6	Fatty acid composition (%TFA) of Artemia cyst from different locations	77
Table 3.7	Fatty acid composition (%TFA) of Artemia nauplii from different locations	79
Table 3.8	Fatty acid composition (%TFA) of Artemia adult from different locations	80
Table 4.1	Generation wise mean nauplii length (µm) in selectively bred Artemia	102
Table 4.2	Heritability estimate and standard error of the Artemia nauplii (first day length)	103
Table 4.3	Selection differential (µm), Phenotypic standard deviation (µm) and Standard Selection differential of the fifteen generations of selected Artemia	104

Table 4.4 Predicted, realized and cumulative gain (µm) in nauplii length of Artemia from fifteen generations of selective breeding 105

Table 4.5 Mean cyst diameter of the base, selectively bred Artemia strains (SBAS) and the different reference Artemia strains... 106

Table 5.1 Total length (TL) of the un-enriched, Algamac enriched and microalgae enriched Artemia nauplii during the nine our post enrichment. 121

Table 5.2 Total width (TW) of the Control and enriched Artemia nauplii during the nine our enrichment study 121

Table 5.3 Mortality Percentage of un-enriched control, Algamac enriched and microalgae enriched Artemia nauplii incubated at 0 ppt., for different durations.. 126

Table 5.4a Fatty acid composition of microalgae (P. viridis and D. inornata) vis-à-vis Artemia nauplii enriched with microalgae 128

Table 5.4b Percentage fatty acid composition of microalgae (N. oculata and I. galabana) vis-à-vis Artemia nauplii enriched with microalgae for up to 9 hour enrichment 129

Table 6.1 Trehalose, Protein and Carotenoid content of Artemia adults under different salinity conditions 156

Table 6.2 Fatty acid content of Artemia adults under different salinity. Conditions... 158

Table 6.3 Amino acid content of Artemia adults under different salinity conditions... 161

List of Figures

Figure No. Title Page No.

Fig. 2.1 Schematic map showing the survey sites in India.................................. 19

Fig. 2.2 A) Artemia cysts float in the corner of a hypersaline water body,.......... 20

B) Collection of Artemia cysts using 50 µm mesh scoop net, ... 20

C) Washing of collected Artemia cyst using 100 µm mesh scoop net,..... 20

D) Microscopic image of individual Artemia cyst 20

Fig. 2.3 A) Stock culture of Microalgae Isochrysis galbanain Hoffkins culture flasks, ... 22

B) Microscopic image of microalgae I. galbana 22

Fig. 2.4 Schematic representation of diallel crossing involving indigenous Artemia strains and Artemia franciscana reference strain 25

Fig. 2.5 Hatching percentage (HP) of the various Artemia strains..................... 30

Fig. 2.6 A) Adult Male Artemia 31

B) Adult Female Artemia 31

C) Sexual breeding Artemia- Pre-copulatory riding pair 31

Fig. 2.7 Mean cyst diameter (µm) of non-decapsulated cysts of indigenous and exotic Artemia populations with standard deviation and one way ANOVA.. 33

Fig. 2.8 Principal Coordinate Analysis (PCoA) of the different strains of Artemia species. PCoA was performed using the software PAST (Palaeontological Statistics) 39

Fig. 2.9 Phylogenetic tree of Artemia constructed by the maximum parsimony analysis using the Internal Transcribes Spacer 1 (ITS-1)...................... 40

Fig. 2.10 Nauplii length and Cyst diameter of different Artemia strains............. 43

Fig. 2.11 Haplotype map of the different Artemia strains collected from various hypersaline habitats of India 47

Fig. 3.1 Schematic map showing the geographical locations of Artemia in Indian subcontinent 60

Fig. 3.2 Trehalose content of Cyst and hatching percentage of the various Artemia strains 68

Fig. 3.3 Mean values of Protein, Trehalose and Carotenoid content of cyst, nauplii and adult stages of indigenous strains of Artemia 82

Fig. 3.4 Mean values of Indispensable Amino acid (IAA) and Dispensable Amino acid (DAA) content of Artemia cyst, nauplii and adult of indigenous Artemia strains 83

Fig. 3.5 Mean values of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) of the Artemia cyst, Nauplii and Adult.................... 86

Fig. 3.6 Mean values of monounsaturated fatty acid (MUFA) content of indigenous and reference Artemia cyst, Nauplii and Adult.................... 87

Fig. 3.7 Mean values of polyunsaturated fatty acid (PUFA) content of indigenous and reference Artemia cyst, Nauplii and Adult.................... 88

Fig. 4.1 Hatching percentage of different Artemia strains 107

Fig. 4.2 Generation wise nauplii length of Artemia under selection.................... 108

Fig. 5.1 (A) Total length and Total width of control Nauplii during the nine hour enrichment 122

(B) Total length and Total width of Algamac enriched Nauplii during nine hour enrichment 122

(C) Total length and Total width of I.galbana enriched Nauplii during nine hour enrichment 123

(D) Total length and Total width of P.viridis enriched Nauplii during nine hour enrichment 123

(E) Total length and Total width of N.oculata enriched Nauplii during nine hour enrichment 124

(F) Total length and Total width of D.inornata enriched Nauplii during nine hour enrichment 124

Fig. 5.2 Mortality percentage of Artemia nauplii during enrichment.................... 125

Fig. 5.3 Mortality percentage of un-enriched control, Algamac enriched and microalgae enriched Artemia nauplii incubated at 0 ppt., for different durations.................... 127

Fig. 5.4 SFA, MUFA and PUFA content of Artemia nauplii enriched with four microalgae 131

Fig. 5.5 DHA (22:6n 3) content in microalgae and the enriched Artemia nauplii.................... 132

Fig. 6.1 *PCR efficiency of β actin, GAPDH and ArHsp22 genes* *153-154*

Fig. 6.2 *Mortality percentage of Artemia subjected to salt stress* *155*

Fig. 6.3 *IAA content of Artemia adults under different salinity conditions (A, B, C, D, E, F, G, H and I)* *162-164*

Fig. 6.4 *Melt curve dissociation analysis of ArHsp22 in graph A, β actin in graph B and GAPDH in graph C* *165-166*

Fig. 6.5 *Normalized expression of ArHsp 22 gene in Artemia under salt stress.* *167*

Chapter 1

General Introduction

Aquaculture is one of the fastest growing animal food producing sectors in the world, accounting for almost half of the total food fish supply. It continues to grow more rapidly than all other animal food production sectors in the world. Production from aquaculture has outpaced population growth, with the annual average per capita supply from aquaculture increasing at the rate 7.1 per cent (FAO 2010). Sustainable larval production is vital to the success of aquaculture. Non availability of healthy, uniform sized, quality seeds in sufficient quantities have always been a concern in 'capture based aquaculture' where seeds are sourced from the wild. This issue could be addressed through the hatchery production of seeds that can ensure the availability of quality seeds in required quantities. The use of hatchery developed seeds may also relieve the pressure on the wild caught seeds. All these have forced the Aquaindustry to move towards 'hatchery based aquaculture' systems (Sadovy de Mitcheson and Liu, 2008).

In hatcheries, live feeds play a key role in deciding the survival, growth and development of the larvae (Sorgeloos *et al.,* 2001). Due to the high growth rate in the early developmental phase of the larvae, the starter diet should hold high levels of soluble proteins, amino acids, carotenoids and fatty acids, especially the *n*-3 and *n*-6 poly unsaturated fatty acids (PUFA). Once the exogenous feeding starts, the growth rate of larvae depends mainly on the availability of suitable, nutrient rich starter diet (Rønnestad *et al.,* 1999).

Live feeds can stimulate the feeding and improve the digestive process of the larvae than the artificial micro diets through its prey-predator interactions and exogenous digestive enzymes (Kamarudin *et al.,* 1994; Baskerville-Bridges and Kling, 2000). Most of the finfish larvae are small with narrow buccal openings, and therefore, exogenous foods of small size are needed for one or two days after hatching (de Barros and Valenti, 2003). Therefore, the success of a starter diet in larviculture lies in its size also rather than its nutrient content alone because the feed has to be perceived, captured, accepted and efficiently ingested by the larvae (de Barros and Valenti, 2003). Among the different live feeds, *Artemia* are widely used as the starter diet in larviculture. Its storage ability, easiness to hatch on demand and their soft texture make them quite essential in marine larviculture (Amat *et al.,* 2005, 2007; Abatzopoulos *et al.,* 2006; Beck and Turingan, 2007). But, the mechanical restriction imposed by the small mouth opening in most finfish and shellfish larvae remains a stumbling block in larval nutrition (Srivastava *et al.,* 2006).

Different *Artemia* sp. are widely distributed on the five continents in the many salt lakes, coastal lagoons and solar salt works. Commercial sources are few, basically there are two: the coastal salt works in the San Francisco Bay (SFB-California, USA) and Great Salt Lake (GSL Utah, USA) (Jean Dhont and Patrick Sorgeloos, 2002). To lessen the dependency on natural *Artemia* sources and reduce the larval rearing expenses, various formulated diets have been tried as alternatives. But, due to reduced preference/acceptability by the larvae, water quality deterioration, etc. their acceptance was low in larviculture (Srivastava *et al.,* 2006).

Although, all stages of *Artemia* are suitable diet for diversified group of finfish and shellfish larvae, nauplii stage is considered as the most preferred larval diet. Hatching efficiency, nauplii size and nutrient profile are the foremost indicators that determine the quality of the *Artemia* of which nauplii size is the most important one in larviculture.

In India, presence of parthenogenetic *Artemia* was first reported by Kulkarni, (1953) from Vadala, Mumbai. Subsequently several researchers reported the occurrence of *Artemia* strains from various part of the country (Baid, 1968; Royan *et al.,* 1970 & 1979; Achari, 1971; Bhargava and Alam 1980; Basil *et al.,* 1987; Kulasekarapandian *et al.,* 1992; John *et al.,* 2004). However, these strains have never been exploited. Instead during the last three decades the Indian aquaculture industry have been importing exotic *Artemia franciscana* strains mainly from San Francisco Bay (California), Great Salt Lake (Utah) in the United States and salt lakes from Saskatchewan in Canada (CMFRI, 2006), to meet its aquaculture needs.

It is suspected that the large scale import of exotic *Artemia* have resulted in the invasion of the Indian waters by them. Invasion and subsequent establishment of exotic species generally pave way for the alteration of ecosystem conditions. Moreover it makes the autochthonous species unfit for life and leads to its extinction which seemed to be one of the major causes for global biodiversity loss. The chance for the extinction of native species is more when the congeneric native species exist in the same niche/ habitat. Therefore, detailed survey for the collection and identification of the indigenous *Artemia* is essential to verify the suspicion. Hitherto, no all-out studies have been carried out to profile the indigenous *Artemia* strains from the different habitats distributed along the Indian subcontinent.

Molecular tools are commonly used for species confirmation as well as to infer relationships among closely related taxa and species. Nuclear genes like ITS 1 and mitochondrial genes such as the large subunit (16S) ribosomal

DNA and cytochrome c oxidase subunit I (COI) are popular markers used in molecular systematic studies of crustaceans at the species and population levels. Among the different molecular markers, ITS-1 that separates the 18S and 5.8S nuclear ribosomal RNA genes has been found to evolve rapidly, and is extensively being used for phylogenetic and population analyses of various groups of animals, such as crustaceans, corals, mollusks, insects and teleosts (Chu *et al.,* 2001; Gondim *et al.,* 2004; White *et al*., 1990). Extensive uses of ITS-1 for estimating phylogenies at different hierarchical levels have been reported by Harris and Crandall, (2000), Weekers *et al.,* (2001) and Chaw *et al.,* (2005). Wide variation in length of the ITS 1 has been reported in various crustaceans (Von der Schulenburg *et a*l., 2001). Chu *et al*., (2001) reported that the high degree of ITS-1 variation among crustaceans above the genus level is the result not only of insertion/deletion events but also of a high degree of substitutions. Hence, Baxevanis *et al.,* (2006) have suggested that apart from the phylogenetic utility of ITS 1, it may also serve as a crude marker for identification of unknown *Artemia* isolates.

Chu *et al.,* (2001) also observed that though the ITS-1 variations are high among different taxonomic groups of crustaceans, variations between congeneric species appear to be genus-specific, making ITS-1 a phylogenetically informative marker in some genera, or a valuable diagnostic tool in others making it a potential marker with applicable in the analysis of crustacean population structure. Hence, we have selected ITS-1 marker for molecular characterization in the present study.

In fact *A. franciscana* is considered as an invasive species around the world as they compete with the native brine shrimp populations due to its superior adaptive capabilities (Amat *et al*., 1991; Amat *et al*., 2007; Ruebhart *et al*., 2008). Though, different *Artemia* strains are found widely distributed in various hypersaline habitats across the globe (Stappen, 2002), based on their nutrient quality, small naupliar size and hatching efficiency, only few strains are used extensively as starter diet in larviculture. Though, *Artemia* strains with higher nutrient qualities are preferred and have great demand in aquaculture, very few attempts have been made to evaluate the nutrient quality and suitability of indigenous *Artemia* strains as live feeds for Indian aquaculture. It is essential to evaluate the nutritional content with respect to soluble protein, indispensable amino acids (IAA), dispensable amino acids (DAA), carotenoids and polyunsaturated fatty acids (PUFAs) along with the life history characters of indigenous *Artemia* strains to determine their suitability. In this scenario, a detailed study of the native *Artemia* strains for different geographical locations of India to evaluate the species representation and phylogenetic status, nutritional content etc., was considered worthwhile.

A key concern in the larval rearing process is the timely availability of small size nauplii. Selection of the diet by the larvae depends on the prey size rather than its nutritional quality. Pepin and Penney, (1997) observed a positive correlation between the mouth widths of the larvae with the prey size. Live feed- larvae interactions have been studied in many fish species (Olsen *et al.*, 1999). Despite having excellent nutritional profile, some *Artemia* strains, say for example *Artemia tibetiana* are not considered as the best larval diet because of their large nauplii (Stappen *et al.,* 2003). Various approaches are being adopted to selectively harvest small size live prey to be used as starter diet for small mouthed larvae. Decapsulated *Artemia* cysts are often used as starter diet to the small size larvae, but biochemical analysis revealed the nutritional inferiority of the cysts when compared to the nauplii stage (García-ortega *et al.,* 1995). Genetic manipulation is an ideal technique to bring about desired change in nauplii size like any other biological trait. Selective breeding is a time tested quantitative genetic manipulation technique employed in plants and livestock, and can be used to develop *Artemia* producing small nauplii.

Information on the selective breeding studies in *Artemia* is scanty. However heritability estimates of hatching percentage, cyst size, growth rate, thermal stability and heat resistance have indicated the superiority of genotypic influence over the phenotypic control on this trait (Tackaert *et al.*, 1987; Clegg *et al.,* 2000; Clegg and Trotman, 2002; Briski *et al*., 2008). Shirdhankar and Thomas, (2003) have reported heritability values of moderate magnitude in *Artemia franciscana* using half-sib analysis. Clegg *et al*., (2000, 2002) reported that the changes in thermal stability and heat resistance occur extremely rapidly using genetic selection in Vietnam strain of *Artemia*. Bi-directional selection in *Artemia franciscana* was reported to be very effective for reduction of nauplii length by Shirdhankar *et al*., (2004) . In this scenario, considering the need for reducing the *Artemia* nauplii size keeping the nutrient content in optimum level selective breeding was attempted in the indigenously collected strain.

Artemia nauplii lack certain essential nutrients required for the marine fish larvae. Hence, it is necessary to meet the essential nutrients requirement, through the enrichment of live feeds. Commercial enrichment diets are widely used for this purpose (Chakraborty *et al.,* 2010; Figueiredo *et al.,* 2009; Biswas *et al.,* 2006; Sorgeloos *et al.,* 1991). As an alternative to the commercial enrichment diets, there is a growing interest in the aqua-industry to use marine microalgae as enrichment diet for the live feeds (Chakraborty *et al.,* 2007). The major problems associated with nauplii enrichment are the incidence of naupliar mortality and rapid growth during enrichment. Though, the enriched nauplii have high nutrient profile, their larger size prevents them from being ingested by the small mouthed fish larvae (Sorgeloos *et al.,* 2001). Incidence of higher naupliar mortality before being ingested by the fish larvae remains another issue since dead nauplii are seldom preferred by fish larvae. Most of the enrichment studies have evaluated the changes in the enriched nauplii, its nutrient content and growth rate mainly using commercial enrichment formulations (Narciso, 2000; Han *et al.*, 2001; Ritar *et al.*, 2004; Figueiredo *et al.*, 2009). In this context, a detailed study on pre and post enrichment effects of microalgae diet on the *Artemia* nauplii is valuable to develop an enrichment protocol and assess their superiority over commercial enrichment formulations.

In aquatic environments, organisms experience different stressors mainly due to the anthropogenic interventions particularly the burning of fossil fuels, which are drastically altering the global climate (Cavallo *et al.*, 2010; Barton and Iwama, 1991), and also due to the natural stressors which formed either the end effect of anthropogenic interventions or as a result of natural calamities (Kelly *et al.*, 2000). According to the Intergovernmental Panel on Climate Change (IPCC), (2007) “Warming of the climate system is unequivocal, as is now evident from observations of increases in global average air and ocean temperatures, widespread melting of snow and ice, and rising global average sea level”. Temperature shifting and salinization of freshwater bodies and hypersalinazation of saline ecosystems are the first and immediate stressors which directly cause to the imbalance of the ecosystem (Cavallo, *et al.*, 2010; Krishnakumar *et al.*, 1991). Since the aquatic organism are in close contact with the medium via their gills, exoskeleton, skin and scales, their stress response are swift than the terrestrial animals. This in turn, negatively affect the animal homeostatic equilibrium, feed conversion ratio (FCR), specific growth rate (SGR), survival, reproductive performance and immune system (Anger 2003; Wendelaar Bonga, 1997).

Brine shrimp, *Artemia* are distributed along the hypersaline, inland salt lakes and salt pans around the globe. They are rarely found in waters with salinity lower than 45 ppt, although physiologically they thrive in seawater and even in brackish waters (Pearson and Sorgeloose, 1980). Salinity is without any doubt the predominant abiotic factor determining the presence of *Artemia* and the natural defense mechanism against predators consequently limiting its geographical distribution (Stappen, 2002). Tackaert & Sorgeloos, (1991) have mentioned a genetically imprinted ecological response in brine shrimp for allowing optimal development among the populations at higher salinities (between 100-180g/l) which is a natural way for the elimination of predators from the local habitats (Rodríguez -Almaraz *et al.,* 2006). According to Triantaphyllidis *et al.,* (1995), El-Bermawi *et al.,* (2004) and Agh *et al.,* (2008) growth rate of *Artemia* is inversely proportional to salinity, and they also observed significant differences in growth rate in Tanggu (China) populations of Parthenogenetic *Artemia* populations when cultured in different salinities. Baxevanis *et al.,* (2004) observed that an increase in both temperature and salinity had negative influence on the overall reproductive performance and morphometric characters, and pointed out that salinity is the prevailing environmental component and the major selective force sculpturing *Artemia* populations. Bowen *et al.,* (1985) and Lenz, (1987) reported that *Artemia* inhabit chloride, sulphate or carbonate waters and combinations of more than two anions in nature. According to Clegg and Trotman, (2002) the challenging ecological settings make *Artemia* a useful model organism for studies on evolutionary and ecological aspects of the stress response, at all levels of biological organization.

During stress, molecular chaperones interact with other proteins to modulate folding, cell localization and functionality, and to protect against irreversible denaturation, and the small heat shock proteins (sHSPs) are noted for their protective capability, representing the first line of defense against physiological and environmental stresses (Qui and MacRae, 2008). According to Tanguay *et al.*, (2004) in the absence of stress, all of the sHSP26 are in the cytoplasm, presumably freely diffusing and not associated with cytoplasmic elements, and upon encountering stresses such as high temperature or anoxia about half of the p26 is translocated to nuclei and probably other sites such as mitochondria and possibly the endoplasmic reticulum. Further, these translocations are apparently initiated by a stress-induced acidification within the cells of the embryo and the translocated p26 is believed to function as a molecular chaperone for proteins while the embryo is under stress and during recovery (Willsie and Clegg, 2002). ArHsp22 is the third sHSP identified in diapause- destined embryos of *A. franciscana*, joining ArHsp21 and the p26 (Qiu & MacRae, (2008); Sun Y & MacRae, (2005); Sun *et al.*, (2006); Clegg (2007); Villeneuve *et al.*, (2006)). It possesses an α-crystallin domain flanked by variable amino-and carboxy-terminals, all with characteristic sequence properties (Sun & MacRae, 2005).

According to Qiu and MacRae, (2008) ArHsp22 inhibits citrate synthase aggregation *in vitro* under elevated temperature and check dithiothreitol-induced denaturation of insulin at 25 ^{0}C, and there is little difference between the *in vitro* chaperone activities of ArHsp22, ArHsp21 and p26. Exhibition of chaperone activity in vitro indicates that ArHsp22, ArHsp21 and p26 contribute to diapause maintenance by enhancing stress tolerance (Qiu and MacRae, 2008).

Different studies of Clegg *et al.*, (1962, 1965, 1974, 1986, 2002, 2004) revealed that trehalose, which constitute 15–17% of dry *Artemia* eggs was involved in the maintenance of the high ultra-structural integrity, and was proved to protect membrane structure and functioned against desiccation damage. Glycerol, which constitute 2–5% of dry egg mass was hypothesized to protect embryos in stress environments (Campagna, 2006). Wang *et al.*, (2010) reported that some biochemical components synthesized in the *Artemia* eggs also play protective roles. Hence the effect of salinity stress on sHSP expression and biochemical constituents such as trehalose, carotenoids, fatty acids, amino acids, protein etc., was also taken up.

The objectives of the present study are as follows:

1) Collection followed by morphological and molecular characterization of *Artemia* populations from different hypersaline habitats of the Indian subcontinent
2) To investigate the extend of invasion and naturalization of exotic *Artemia* strains in Indian Salinas and assess the displacement of the native parthenogenetic *Artemia* from its natural habitats
3) To explore the potential *Artemia* strains with small naupliar size and higher nutritional content from various geographical locations of India
4) To develop a small nauplii producing *Artemia* by selective breeding and to compute the heritability estimates of the selected *Artemia*
5) To find out the effect of microalgae and commercial diet on the survival and growth rate of *Artemia* nauplii at different time intervals and the stress tolerance of the post enriched nauplii subjected to different saline conditions
6) To study the effect of salinity stress on a) survival rate, b) biochemical constituents such as soluble protein, amino acid, trehalose and fatty acid and c) the real time expression of ArHsp 22 in allochthonous *Artemia* collected from Indian Salinas.

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Chapter 2

Molecular and morphologic characterization of Brine Shrimp, *Artemia sp.*

P. A. Vikas, N. K. Sajeshkumar, P. C. Thomas, Kajal Chakraborty and K. K. Vijayan. (**2012**)
Aquaculture related invasion of the exotic *Artemia franciscana* and displacement of the autochthonous *Artemia* populations from the hypersaline habitats of India. ***Hydrobiologia***, 684:129–142.

2.1. Abstract

An extensive survey was conducted to study the *Artemia* populations from the various hypersaline habitats of the Indian subcontinent. The study revealed the presence of *Artemia* in North West (Mithapur & Nanganvadi), South West (Tamaraikkulam & Tuticorin) and South East (Kelambakam & Marakkanam) regions. The *Artemia* populations in all these locations were sexually breeding, indicating the invasion by sexual populations displacing the parthenogenetic populations earlier reported from the Indian habitats. Individual culture experiments revealed the absence of parthenogenesis, and crossbreeding experiments proved the sexual mode of breeding in these populations. One commercial strain of *Artemia franciscana* (Great Salt Lake origin) and six geographical strains of the naturalized *Artemia franciscana* from the different habitats of India were evaluated for their cyst biometry and life history traits. The study revealed that the size of the cysts varied from 219.6 to 236.4μm and

that of nauplii from 496.0 to 514.8µm, and Marakkanam strain (TMM) possessed the smallest cysts (219.6µm) as well as nauplii (496.0µm). Estimates of other life history traits namely, the third-day length, sixth-day length, length of male at sexual maturity and female length at sexual maturity was comparable with other reported *Artemia* species/strains. The differences among the strains were significant. Among the Indian strains, Mithapur strain (GMJ) had the highest hatching percentage (84.5%), and was found to be superior to even the commercial strain (SFB) which had only 72.2% hatching percentage. Internally Transcribed Spacer-1 (ITS-1) from the Indian population was PCR amplified and sequenced. The ITS-1 sequence of the Indian *Artemia* populations exhibited 99% homology with the exotic *Artemia franciscana.* The mean pair-wise genetic distances between the Indian *Artemia* populations were negligible, indicating their genetic similarity. The absence of any significant genetic distance values between Indian *Artemia* populations and *A. franciscana* confirms that they are conspecific. Phylogenetic analysis grouped all the Indian *Artemia* populations with *A. franciscana* species. Morphological and biometric observations, crossbreeding experiments, molecular data and phylogenetic analysis confirms that the Indian *Artemia* populations are genetically similar to *A. franciscana.* Lack of regional endemism in populations distantly located was evident, indicating that they have naturalized and are in the process of evolution. Principal Coordinate Analysis (PCoA) clearly grouped the Indian *Artemia* populations and the reference strain of *A. franciscana* in to a single cluster. While the remaining new world species viz, *A. persimilis*, *A. salina* and *A. sinica* were grouped into another clade, the old world *A. persimilis* stood alone separately. Widespread import and use of alien *A. franciscana* as live feed in hatcheries must have paved the way for its massive invasion followed by the displacement of *parthenogenetic Artemia* from the Indian hyper-saline habitats.

This forms the first report of invasion by *A. franciscana* in the hyper-saline habitats of Indian subcontinent and further studies are required to assess the biological implications of this invasion.

2.2. Introduction and review of literature

Live feeds possess an edge over the formulated larval diets, and play a vital role in the growth and development of finfish and shell fish larvae in hatcheries. Most of the finfish larvae are small and have small buccal openings. (deBarros and Valenti, 2003). The mechanical restriction imposed by the small mouth opening in most finfish and shell fish larvae remains a stumbling block in larval nutrition (Srivastava *et al.*, 2006). The success of a starter diet in larviculture lies firstly in its size rather than its nutrient content because the feed has to be perceived, captured, accepted and efficiently ingested by the larvae (de Barros and Valenti, 2003). Selection of the diet by the larvae depends on the prey size rather than its nutritional quality. Pepin and Penney, (1997) observed a positive correlation between the mouth widths of the larvae with the prey size.

Artemia nauplii possess an edge over the formulated diets and other live feeds due to their higher acceptability, texture and nutrient profile. Though *Artemia* is not the natural prey of many aquatic animals, they have become a ready choice due to the factors such as easy storage in cyst form and ability to produce nauplii at the desired place and time. A key concern in the use of *Artemia* in larval rearing process is the availability of small size nauplii. Despite having excellent nutritional profile, some *Artemia* strains are not considered as the best larval diet because of their large sized nauplii as in *Artemia tibetiana* (Stappen *et al.*, 2003).

Although, *Artemia* have been reported from the Indian Salinas they have not been properly evaluated. In fact their evaluation, especially their morphometric features is as important as its nutrient evaluation to identify suitable strains for use as live feeds for Indian aquaculture. The few studies on the indigenous *Artemia* have reported that they reproduce parthenogenetically and belong to the species *Artemia parthenogenetica.*

They were reported to be of larger size making them less preferable compared to exotic species. The studies have indicated that it has larger cyst and nauplii size (244.9 ± 7.4 µm and 492.8± 21.4 µm respectively) compared to exotic species. Therefore, *Artemia* from the Indian Salinas have not been exploited for commercial use, and during the last three decades the Indian aquaculture industry have been importing exotic *Artemia franciscana* strains mainly from San Francisco Bay (California), Great Salt Lake (Utah) in the United States and salt lakes from Saskatchewan in Canada (CMFRI 2006), to meet its aquaculture needs. It have been suspected that widespread import and use of alien *A. franciscana* as live feed in hatcheries must have paved the way for its massive invasion in Indian habitats.

The principal components of human-induced alterations to aquatic ecosystems are the introduction of exotic animals and loss/alteration of habitats resulting from aquaculture and related activities. An exotic invasive species is one, which if introduced will become established and inflict damage on the biodiversity, ecosystem integrity, agriculture, fisheries, and public health (Allendorf & Lundquist, 2003). Many native aquatic species are threatened by competition and predation from invaders, and the invasive species are a leading cause of animal extinctions, resulting in ecosystem modification and loss of biodiversity (Mooney & Cleland, 2001; Gurevitch & Padilla, 2004; Clavero & García-Berthou, 2005; Vijayan *et al.*, 2004 & 2009). Apart from their substantial economic and environmental impacts, invasive species may alter the evolutionary trajectory of natives through competition, displacement, hybridization and even extinction (Sakai *et al.*, 2001) and is especially detrimental for a variety of continental aquatic invertebrates with dormant life stages and similar morphology (Muñoz, 2010).

The brine shrimp *Artemia* (Branchiopoda, Anostraca) is widely distributed in the salt pans, inland salt lakes and hyper-saline water bodies throughout the world (Royan, 1970; Stappen, 2002). It comprises of a group of sexual and parthenogenetic species probably diverged five to six million years ago from an ancestral form living in the Mediterranean area (Triantaphyllidis *et al.*, 1998; Abatzopoulos *et al.*, 2002). Parthenogenetic *Artemia* is distributed over vast geographic areas, of the continents of Australia, Europe, Asia and Africa (Gajardo *et al.*, 2002; McMaster *et al.*, 2007). In contrast, sexual *Artemia* species are geographically restricted (Muñoz *et al.*, 2010). In India, the occurrence of native parthenogenetic *Artemia* populations was first reported from Vadala, Mumbai (Kulkarni, 1953). Subsequently, many researchers reported the occurrence of this strain from various regions of the Indian subcontinent (Baid, 1968; Royan, 1970 & 1979; Achari, 1971; Bhargava & Alam, 1980; Basil *et al.*, 1987; Kulasekharapandian *et al.*, 1992; John, 1994).

Artemia nauplii have been used as live feed for over 85 percent of the fin fish and shell fish larvae (Chakraborty *et al.*, 2010).The first recorded deliberate introduction of *A. franciscana* for aquaculture purpose was on Pacific Island in Brazil in the 1970s (Stappen, 2002). According to Abatzopoulos *et al.*, (2002) *A. franciscana* was first established in the North American region. Amat *et al.*, (2005) have remarked that *A. franciscana* is an exotic invasive species in the western Mediterranean countries including the Atlantic shore salterns in Portugal and southwest Spain, France, as well as in the north of Morocco. Presently *A. franciscana* is considered as an invasive species around the world as they compete with the native brine shrimp populations due to its superior adaptive capabilities (Amat *et al*., 1991; Amat *et al*., 2007; Ruebhart *et al*., 2008). In India, exotic *Artemia* cyst introduction was started in early 1980's to be used as live feed to use in finfish and crustacean hatcheries and commercial culture ventures (Kulasekarapandian *et al*., 1995). During the last three decades the Indian aquaculture industry have been importing exotic *A. franciscana* strains mainly from San Francisco Bay (California), Great Salt Lake (Utah) in the United States and salt lakes from Saskatchewan in Canada (CMFRI 2006).

Studies of Elton, (1958) initiated the unraveling the impact of invasive species on the ecosystems, communities and native species is known since several decades ago. Amat *et al.,* (2007) reported that the level of knowledge and understanding of the mechanisms leading to the success of the invasive is far to provide neither criteria to predict the threat of invasions nor how to act or to manage ecosystems already invaded. Success of the invasion and its further establishment in natural biota depends on the competitive and trophic interactions of the invading exotic and analog native species. Invasive species usually have an edge over the native species for food and space and often remain at low levels for an extended period of time before being fully established. They slowly colonize the habitats, become naturalized and eventually replace the native species in the course of time. This phenomenon has a global importance with a huge biodiversity impact particularly in aquatic ecosystems (Ruiz *et al*., 1999). The chance for the extinction of native species is more when the congeneric native species exist in the same niche/ habitat. Invasion and subsequent establishment of exotic species generally pave way for the alteration of ecosystem conditions. Moreover it makes the autochthonous species unfit for life and leads to its extinction which seemed to be one of the major causes for global biodiversity loss.

Molecular tools are commonly used to infer the relationships among closely related taxa and species. Mitochondrial genes such as the large sub-unit (16S) ribosomal DNA and cytochrome c oxidase sub-unit I (COI) are popular markers used in molecular systematic studies of crustaceans at the species and population levels. Among the different molecular markers, Internally Transcribed Spacer-1 (ITS-1) that separates the 18S and 5.8S nuclear ribosomal RNA genes has been found to evolve rapidly, and are being extensively used for phylogenetic and population analyses in various groups of animals, such as crustaceans, corals, mollusks, insects and teleosts (Chu *et al.*, 2001;Gondim *et al.*, 2004; White *et al.*, 1990).

Apart from the mode and rate of evolution of ITS-1, it has been extensively used for estimating phylogenies at different hierarchical levels (Harris and Crandall, 2000; Weekers *et al.*, 2001; Chaw *et al.*, 2005). Wide variation in length of the ITS-1 has been reported in various crustaceans (Von der Schulenburg *et al.*, 2001). Chu *et al.*, (2001) reported that the high degree of ITS-1 variation among crustaceans above the genus level is the result not only of insertion/deletion events but also of a high degree of substitutions. Hence apart from the phylogenetic utility of ITS-1, it may also serve as a crude marker for identification of unknown *Artemia* isolates (Baxevanis *et al.*, 2006).

Chu *et al.*, (2001) also observed that the ITS-1 variations are high among different taxonomic groups of crustaceans, variations between congeneric species appear to be genus-specific, making ITS-1 a phylogenetically informative marker in some genera, or a valuable diagnostic tool in others and ITS-1 is potentially applicable in the analysis of crustacean population structure. Hou *et al.*, (2006) have observed those variations in morphometric characters and the genetic variability of Internal Transcribed Spacer -1 (ITS-1) sequence can be used as tools in taxonomic and phylogenetic studies. Hence, we have selected ITS-1 marker for the molecular characterization in the present study.

The present study was undertaken with the aim of (a) morphologic and molecular characterization of the *Artemia* populations in the Indian subcontinent, (b) species identification to investigate the extend of invasion and naturalization by the exotic *A. franciscana* in Indian Salinas and (c) assess the displacement of the native *A. parthenogenetica* from its natural habitats.

During the present study, morphological and biometric observations, crossbreeding experiments, molecular profiling of ITS-1 gene and phylogenetic analysis of the geographical strains of *Artemia* from different habitats of India were used for achieving the above objectives.

2.3. Materials and methods

An extensive survey was conducted in the Salinas of the coastal states of Indian sub-continent, both on the East (West Bengal, Orissa, Andhra Pradesh and Tamil Nadu) and West regions (Maharashtra, Gujarat and Rajasthan) for the presence of *Artemia* populations. *Artemia* samples were collected from different natural hypersaline habitats along the North West (Mithapur- GMJ & Nanganvadi-GNM), South West (Tamaraikkulam-TNM & Tuticorin- TTJ) and South East (Kelambakam-CKF, Vedaranyam-VDA & Marakkanam-TMM) coasts of the Indian subcontinent during 2008 to 2010 (Table 2.1, Fig.2.1). Adult individuals were collected using scoop nets (0.1mm and 1mm mesh) and packed with oxygen in polyethylene bags; cysts were packed in polyethylene bags with hyper-saline brine (250‰) and transported to the wet laboratory of Central Marine Fisheries Research Institute, Cochin for further studies (Fig.2.2). Adults were maintained in acrylic tanks (50 L), while cysts were cleaned and processed by biphasic flotation method (Sorgeloos *et al.*, 1986) and kept at 4ºC until analysis. In addition, *A.*

franciscana strain from San Francisco Bay (SFB) procured from commercial suppliers and strains of *Artemia tibetiana* (TBS) and *Artemia salina* (ASL)received as a gift from the Laboratory of Aquaculture & *Artemia* Reference Center, Faculty of Bioscience Engineering - Department of Animal Production, Ghent University, Belgium were also used in the study.

Fig 2.1 Schematic map showing the survey sites in India.

Black spot with white letters indicate the presence and pentagon transparent shape with black alphabets indicate the absence of *Artemia* in that location.

Fig. 2. 2 A) *Artemia* cysts float in the corner of a hypersaline water body
B) Collection of *Artemia* cysts using 50 μm mesh scoop net
C) Washing of collected *Artemia* cyst using 100 μm mesh scoop net
D) Microscopic image of individual *Artemia* cyst

Table 2.1 Collection locations, date of collection, Geographical Coordinates, Nature of Sample and Gender of the *Artemia* samples collected from Indian Salinas

Location/Collection site	Date of collection	Geographical Coordinates	Nature of Sample	Gender
Kelambakam, Tamil Nadu, India	02-‘08	12^{0}47’ N - 80^0 13’ E	*Artemia* Live & Cyst	Female & Male
Mithapur, Gujarat, India	06-‘08	22^0 24’ N - 69^0 01’E	*Artemia* Live & Cyst	Female & Male
Nanganvadi, Gujarat, India	03-‘09	23^0 07’ N - 70^0 41’E	*Artemia* Live	Female & Male
Marakkanam, Tamil Nadu, India	03-‘09	12^{0}12’ N - 79^0 56’ E	*Artemia* Live & Cyst	Female & Male
Thamaraikulam, Nagercoil, India	02-‘09	08^0 06’ N - 77^0 29’E	*Artemia* Cyst	Female & Male
Tuticorine, Tamil Nadu, India	06-‘08	08^{0}46’ N - 78^0 08’ E	*Artemia* Live & Cyst	Female & Male
Vedaranyam, Tamil Nadu, India	04-‘08	10^0 22’ N - 79^0 51’ E	*Artemia* Live & Cyst	Female & Male

2.3.1. Hatching of cyst

Artemia cysts were hydrated and hatched as described by Vanhaecke and Sorgeloos, (1980). *Artemia* cysts (1gm) were hydrated in 50 ml freshwater for 2 hour duration and the hydrated cyst were collected using PVC nylon filter of 100 µm mesh size. Hydrated cysts were treated with 0.5gm active chlorine (sodium hypochlorite) 40% sodium hydroxide (0.33 ml) and made up to 15 ml with seawater. Decapsulated cysts were filtered and washed thoroughly using fresh water to remove all traces of hypochlorite and incubate at 25± 1.5^{o}C in seawater (35 ppt) under florescent light (1500 lux) for hatching. Freshly hatched *Artemia* nauplii were harvested and stocked in acrylic tanks (40 L) with seawater of 50‰ salinity at 25 ± 1.5 ^{0}C. Nauplii were fed with microalgae, *Isochrysis galbana* (30 X 10^4 cells ml^{-1}) *ad libitum* from a microalgae culture maintained at the laboratory under mild aeration at a 12D:12L photoperiod.

2.3.2. Microalgae culture for *Artemia* rearing

Stock culture of microalgae *Isochrysis galbana* required for feeding the *Artemia* were taken from the marine microalgae culture facility of CMFRI, Cochin. Microalgae were cultured in Hoffkin's glass flasks (3 L) under circadian light: dark cycle (12 h: 12 h) with a light intensity of 162µmol photon $m^{-2}s^{-1}$ at a temperature of 23°C. Each culture flask contained (Fig.2.3) culture medium (2.5 L) prepared with autoclaved seawater (35‰) enriched with the Walnes nutrients (KNO_3, 0.99mM; Na_2HPO_4, 0.11mM; EDTA, 0.12mM; H_3BO_3, 0.54mM; $FeCl_3.6H_2O$, 4.81µM; $MnCl_2.4H_2O$, 1.82µM; $ZnCl_2$, 30.82mM; $CoCl_2.6H_2O$, 16.81mM; $CuSO_4.5H_2O$, 16.02mM; $(NH4)6MO_7O_{24}.4H_2O$, 1.46mM; thiamin,1g L^{-1} and vitamin B_{12}, 50mg L^{-1}) (Walne, 1970). The cell density of microalgae culture were assessed by counting cells in a Neubauer Haemocytometer under microscope (Leica, USA), and inoculated at a cell density of 7×106 cells ml^{-1} and grown up to stationary phase. These stocks cultures were further mass cultured with autoclaved seawater (35‰) enriched with modified Walnes Medium as stated earlier in plastic buckets (20 L) with continuous aeration.

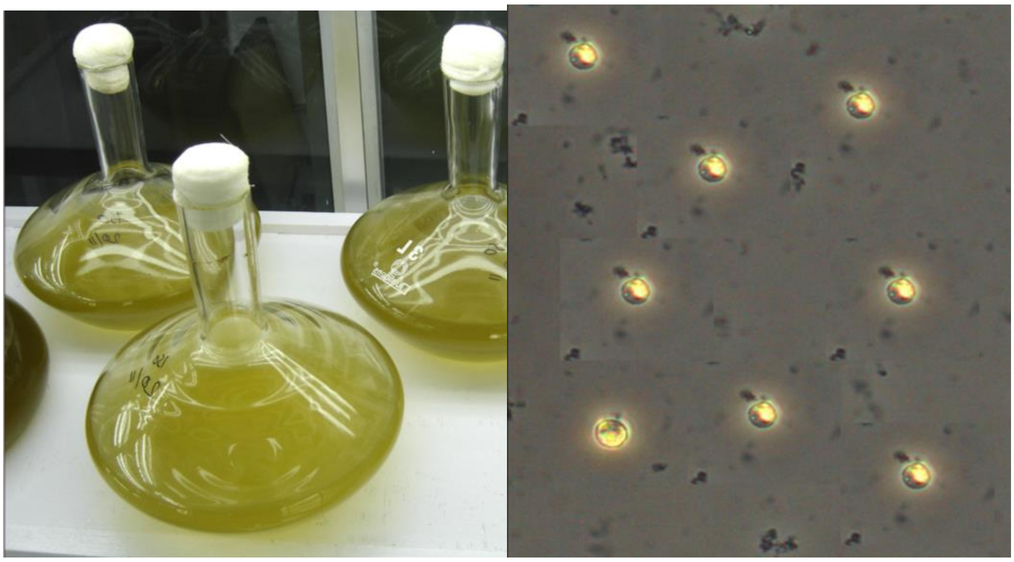

Fig. 2.3.A) Stock culture of Microalgae *Isochrysis galbana* in Hoffkins culture flasks, B) Microscopic image of microalgae *I. galbana*

2.3.3. Evaluation of cyst hatching percentage

Hatching percentage of the native strains and reference strain was evaluated following the individual cyst hatching experiment. In short, the cyst of indigenous strains (CKF, VDA, GMJ, TMM, TNM, and TTJ) and reference strain (SFB) were individually incubated for hatching in 96 well micro plates containing seawater (35‰). The hatching plates were incubated at room temperature under light (Sorgeloos, 1986) and were observed hourly for nauplii, after 8 hours of incubation. Hatching percentage (HP) of the cysts was calculated from the data as follows:

Hatching percentage (%) = (number of nauplii hatched /total number of cysts) X 100.

2.3.4. Morphological characterization

Native strains of *Artemia* collected from different geographical locations and the reference strain were examined for the morphological characters. For this, cysts were hatched and the freshly hatched nauplii were harvested and stocked in to *Artemia* rearing tanks (20L) and representative samples were fixed in Lugol's iodine and measured the nauplii length/ first day length (FDL)using a microscope (Leica, Wetzlar, Germany) attached with DIGI EYE 330 camera with the Dewinter software Biowizard. Simultaneously, *Artemia* nauplii were reared in individual culture tanks till maturity following the standard culture method as described in section 2.3.1 above. From these individual cultures samplings were done periodically and measured the third day length (TDL), and sixth day length (SDL) using a microscope (Leica, Wetzlar, Germany) attached with DIGI EYE 330 camera with the Dewinter software Biowizard,and length of male at sexual maturity (LMSM) and length of female at sexual maturity (LFSM) using a compound microscope.

In addition, other life history and reproductive traits were also recorded for which adult *Artemia* individuals were randomly collected from the culture tanks, and fixed using formaldehyde (10%) in small petri dish. Thereafter, they were examined to detect males with distal penis if any, using stereozoom microscope (Leica, Wetzlar, Germany) and to confirm the occurrence of sexual dimorphism.

Cysts biometry of indigenous strains and reference strain were studied. For this, cysts were incubated in fresh water at 28^{0}C and after 2h of hydration they were fixed with Lugol's Iodine solution. The diameter (µm) of 500 representative cyst samples from each location was determined using a microscope (Leica) with DIGI EYE 330 camera attachment andDewinter software ‘Biowizard’.

Mean and standard deviation were estimated for each strain, and one way ANOVA was carried out using SPSS 13.0 programme (SPSS Software, Chicago, USA).

2.3.5. Reproductive pattern

For assessing the reproductive pattern (parthenogentic/sexual reproduction) of the native strains, cysts of CKF, VDA, TTJ, TMM, GMJ, TNM collected from different hyper-saline habitats and reference strain (SFB) were hatched out following the established method as described earlier. The freshly hatched individuals of each strain was harvested and transferred to different rearing tanks (50Lr acrylic) with sea water of 50‰ salinity at 25 ± 1.5 ^{0}C. Nauplii were fed with microalgae, *Isochrysis galbana* (30 X 10^4 cells ml^{-1}) *ad libitum* from a microalgae culture maintained at the laboratory. From every strain, 500 numbers of eight day old juveniles were collected and reared individually in plastic bottles (250mL). The animals were maintained as per the standard rearing methods and observed daily until death (3 to 4 months) to identify the parthenogenetic mode of reproduction, if present.

2.3.6. Crossbreeding experiments

Experiments were conducted to test the breeding efficiency and reproductive performance of the collected populations. For this the cysts of CKF, VDA, TTJ, GMJ, TNM, TMM and reference SFB strains were hatched out and grown for 10 days in separate tanks. Juveniles were sexually segregated and maintained in plastic containers (3L) for seven days to condition the matured males and females. Thereafter, diallel crossing of all these strains were arranged. Commercial strain of *Artemia franciscana* was also used for the cross. Mature males (500 individuals) from each group were maintained with mature females (500 individuals) from every other group separately in 49 culture tanks (5L) until pre-copulatory riding pairs were formed (Fig.2.4). From each tank, 25 riding pairs were selected and maintained separately in 250 mL containers. Each container was observed daily and the cysts and hatched nauplii, if any, were counted and morphometric measurements taken.

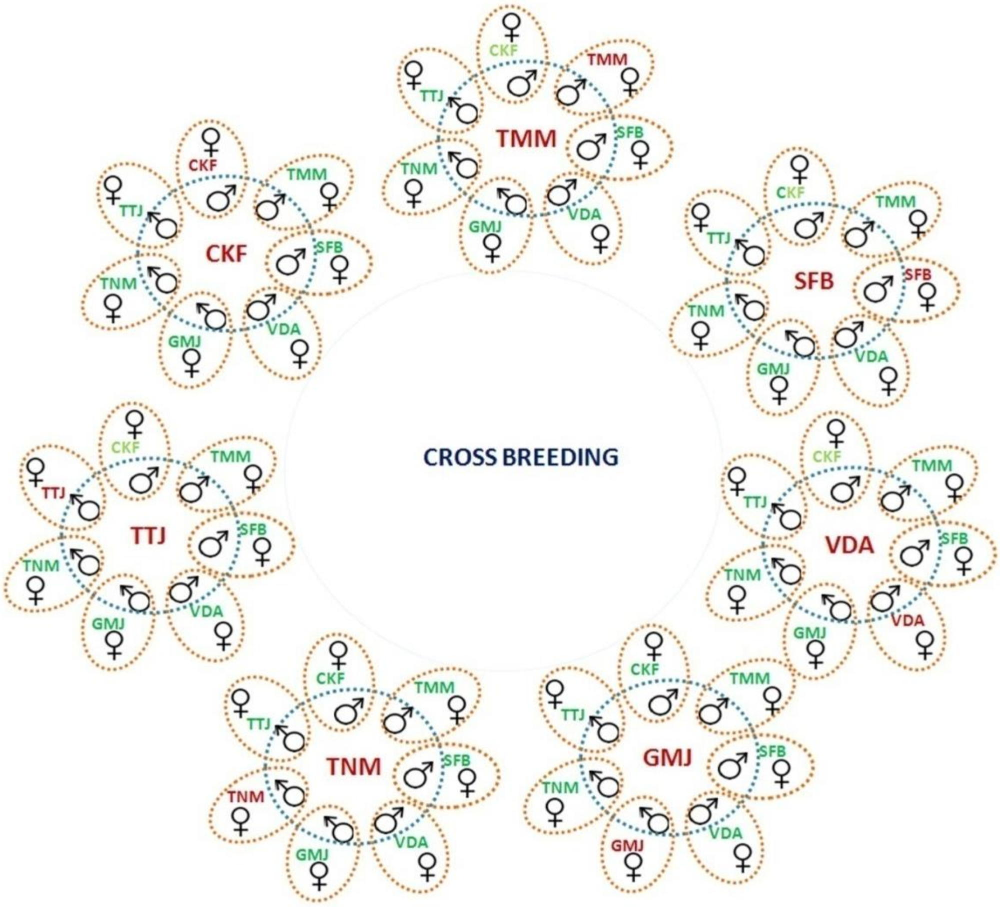

Fig. 2.4 Schematic representation of diallel crossing involving indigenous *Artemia* strains and *Artemia franciscana* reference strain (males of every strain is crossed with females of all other strains).

Further, to assess the phenotypic variations in the cross progenies, the nauplii were reared until maturity and life history traits such as first-day length (FDL), third-day length (TDL), sixth-day length (SDL), length of male at sexual maturity (LMS) and length of female at sexual maturity (LFS) were recorded. Mean, standard deviation and one way ANOVA was performed using SPSS 13.0 program (SPSS Software, Chicago, USA).

2.3.7. Molecular genetic characterization

Molecular characterization of the indigenous strains was carried for species confirmation and other phylogenetic analyses. For this the Internally Transcribed Spacer-1 (ITS-1) from the Indian strains were PCR amplified, sequenced and compared with different *Artemia* species. The genetic distances were estimated and phylogenetic tree constructed. An account of the materials and methods used for the molecular characterization work is presented below.

2.3.7.1. DNA isolation

Total genomic DNA of individual adult *Artemia* was isolated following the procedure described by Miller *et al.* (1988).

Reagents used:

1. 0.5M TrisHCl (pH-8.0) : Tris base and HCl
2. 0.5M EDTA (pH-8.0) : EDTA and NaOH.
3. 10mM TrisHCl (pH-7.5) : Tris base and HCl
4. RNAase buffer : 10mM TrisHCl and 15 mMNaCl

Working Solutions:

1. Lysis buffer : 50mM Tris-HCl (pH8.0) + 20mM EDTA (pH8.0) + 2% SDS

2. Saturated Sodium Chloride : 6M NaCl solution
3. Proteinase K : Proteinase K (20mg/ml)
4. TE buffer : 10mM TrisHCl (pH-8.0) + 1mM EDTA (pH-8.0)
5. RNAase : RNAase (10 mg/ml)

DNA was extracted from the different indigenous strains as well as the reference strains for PCR amplification of ITS-1. The *Artemia* adults were washed thoroughly in freshwater and starved for 24 in autoclaved seawater. Thereafter, *Artemia* samples were homogenized with lysis buffer (500µl) taken in sample vial (1.5ml). Homogenate was then mixed with 5 µl of Proteinase K (20mg/ml) and incubated at 55^0C in water bath for 2 hours for digestion. Fully digested samples were kept in ice for 10 minutes and mixed with saturated NaCl solution (250 µl). Samples were centrifuged (8000 rpm for 15 minutes) and the supernatant transferred to fresh tube (1.5 ml). To this 1.5µl RNase (final conc.20µg/ml) were added and incubated at 37°C for 15 minutes using a heating block. To this double the volume of (1Ml) ethanol (Merck, Germany) was added and incubated at -20°C overnight to precipitate DNA. Thereafter the samples were centrifuged at 11000rpm for 15 minutes to pellet the DNA. The pellet was washed using ice cold 70% ethanol (250µl) by centrifugation (11000rpm for 5 minutes). The supernatant was discarded and the DNA pellet was air dried at room temperature and re-suspended in TE buffer (50 µl). DNA was quantified using a BioPhotometer (Eppendorf, Hamburg, Germany) and stored at -20^0C until further use.

2.3.7.2. Amplification and sequencing of ITS-1

The DNA segment comprising the partial segment of the 18S ribosomal RNA gene, complete ITS-1, and 5.8S ribosomal RNA gene was amplified using forward primer PTF (5'GGAAGTAAAAGTCGTAACAAGG 3') (Tang *et al.*, 2003) and reverse primer PTR (5' GCCAAGTGATCCATCCTCCAG 3') (Hou*et al.*, 2006). PCR amplifications were performed on a PTC-100® Peltier thermal cycler (Perkin Elmer Gene Amp PCR system 2400, USA). The total reaction volume (25 µL) consisted of 1X assay buffer (100mM Tris, 500mM KCl, 0.1% gelatin, pH 9.0) supplemented with MgCl2 (1.5mM) (Sigma Aldrich), template DNA (20 ng), 10 pM of PTF and PTR primers, 200 µM of each dNTPs (Finzyme, USA), and 1.5 U of Taq DNA polymerase (Sigma Aldrich). Thermal cycling conditions were as follows: initial denaturation at 95^0C for 4 min followed by 35 cycles of 94^0C 30 sec, 59^0 C 45 sec, 72^0 C 45 sec and a final extension of 7 min at 72^0 C. The amplified PCR products were resolved through agarose gel electrophoresis (1.2%) and documented using gel documentation system and cleaned up using a PCR product purification kit (HiMedia Laboratories Pvt. Limited, Mumbai, India.). The purified PCR products were sequenced by dideoxy chain-termination method (Sanger *et al.*, 1977) using the AB 3730 XL capillary sequencer (Applied Biosystems, USA).

2.3.7.3. Sequencing and Phylogenetic analysis

The sequences were analyzed using Gene Tools (Syngene, Cambridge, UK) software. Sequences were aligned using the CLUSTAL-W algorithm (Thompson *et al.*, 1994) in Bioedit 7.0 (DNA Sequence Analysis Software 224 package) (Appendix). Nucleotide sequence characteristics, nucleotide diversity and haplotype diversity were estimated using DNAsp version 4.0 (Rozas *et al.*, 2003). Principal Coordinate Analysis (PCoA) was performed using the software PAST (Palaeontological Statistics) (Hammer *et al.* 2001). Pair-wise genetic distances between the collected samples were calculated based on Kimura 2 parameter model (Tamura *et al.*, 2007).

Phylogenetic tree was constructed with the sequence of the 18S ribosomal RNA gene (partial sequence), ITS-1 (complete sequence) and 5.8S ribosomal RNA gene (partial sequence) from the present study (Gen Bank Accession Nos. GU323289-GU323317) and other reference sequences taken from Gen Bank database (Accession Nos.FJ004942-FJ004946, FJ004923-FJ004924, DQ084193-DQ08419, DQ084193, DQ084198, DQ069927-DQ069928, DQ201308-DQ201309, DQ201269 -DQ201270, FJ004929, and DQ201276). To root the phylogeny, Fairy Shrimp (*Streptocephalus proboscideus)* 18S ribosomal RNA gene-partial sequence; internal transcribed spacer 1- complete sequence; and 5.8S ribosomal RNA gene-partial sequence (AY519840) were used (Maeda-Martınez *et al.*, 1995; Daniels *et al.*, 2004; Baxevanis *et al.*, 2006). Phylogenetic analysis was carried out using both maximum parsimony (MP) and neighbor joining (NJ) method with the software MEGA version 4.1 (Tamura *et al.*, 2007). The heftiness of the phylogenetic tree branches was assessed with 1000 bootstrap pseudo-replicates.

2. 4. Results

The survey revealed that *Artemia* populations were present only in seven locations out of the many surveyed. They were Mithapur and Nanganvadiin Gujarat, Tamaraikulam, Tuticorin, Vedaranyam, Kelambakkam and Marakanam inTamilnadu. *Artemia* could not be observed in Rajasthan (Sambhar and Didhwana), Maharashtra (Vadala, Uran and Thane), Andhra Pradesh (Nellur, Ramatheertham and Iskapalli) Orissa (Ganjam, Humma, Sumadi, Suran and Model salt farm) and West Bengal (Contai/Kanthi, Digha and Sankarpur) (Table.2.1).

2.4.1. Hatching percentage

Hatching percentage (HP) of the various *Artemia* strains showed significant differences (Fig. 2.5). Among the indigenous strains, GMJ strain showed high HP (84.5%) compared to the reference SFB strain (72.2%) and other native strains evaluated SBAS (64.6%), TNM & VDA(55%), CKF (54.4%), TMM (52.4%) and TTJ (45.0) respectively.

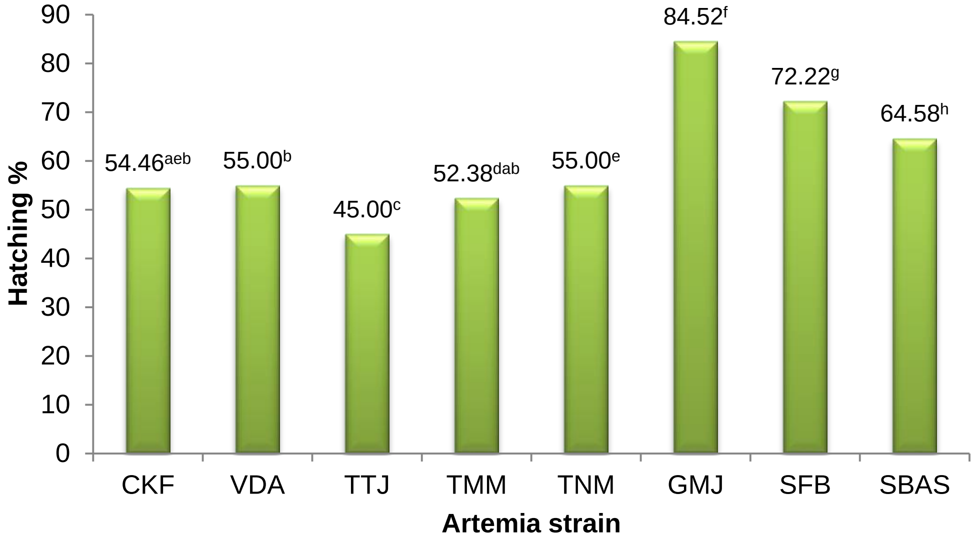

Fig. 2.5 Hatching percentage (HP) of the various *Artemia* strains

2.4.2. Morphological observations

Morphological observations revealed the presence of males with large second antennae (claspers) and distal penis, and females with brood pouches in all the *Artemia* strains collected from various hypersaline habitats of India (Fig. 2. 6).

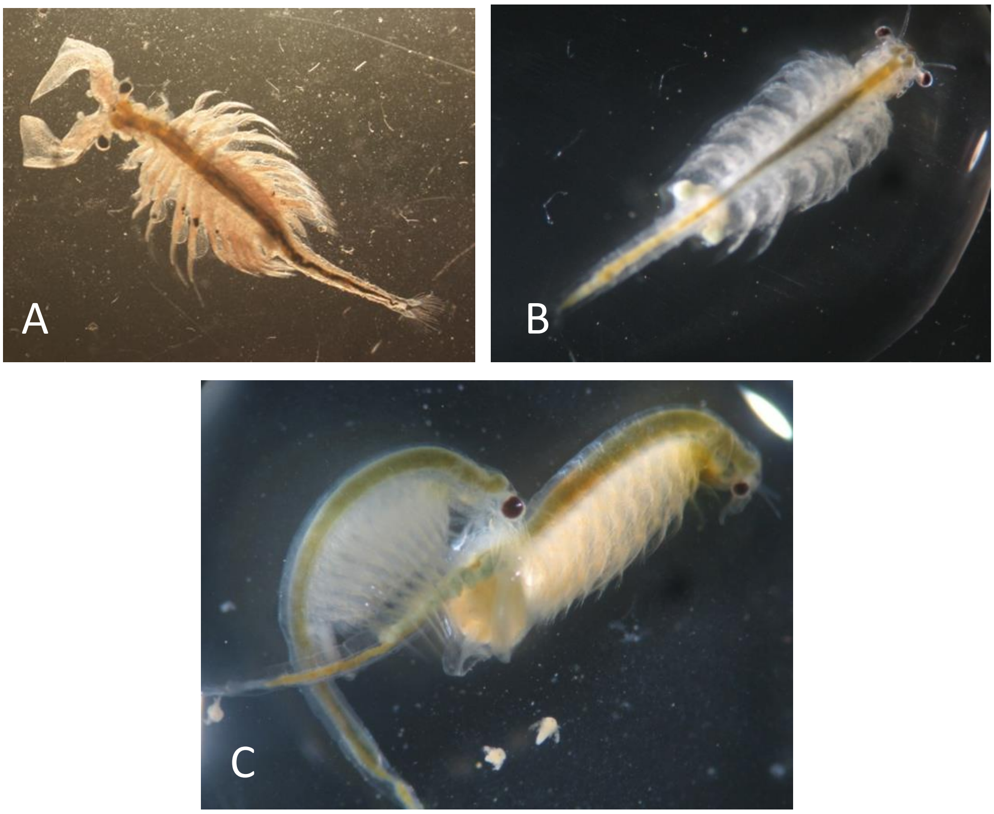

Fig. 2. 6 A) Adult Male *Artemia* B) Adult Female *Artemia* C) Sexual breeding *Artemia*- Pre-copulatory riding pair

Different *Artemia* populations showed significant difference in Average number of Nauplii at first hatch, First day length, Third day length, Sixth day length, Length of male at sexual maturity and Length of female at sexual maturity (Table 2. 2). VDA strain produced the highest number of nauplii at first hatch (80±67) followed by TTJ (61±11), CKF (55±43), SFB (55±24), TNM (55±18), TMM (55±22) and GMJ (54±08). FDL of different *Artemia* strains ranged from 501.0 to 518.0 µm. Among these strains, CKF showed highest FDL (518.0±144.3 µm) and TMM the lowest (501.0±75.76 µm) while in others it was within this range. The strains showed significant difference in TDL, and among the strains TTJ showed the highest TDL (1453±316 µm) followed by SFB (502.6±97.13 µm) and GMJ (1411.±269.4 µm).In the case of

SDL, LMS and LFS the difference among the various indigenous strains and with the reference strain were not significant.

Table 2.2 Mean values of life history traits of the geographical strains of indigenous *Artemia*

Female	ANFH	FDL	TDL	SDL	LMS	LFS
CKF	55±43	518.0 ± 144.3^{a}	$1153.\pm146.0^{ab}$	3.5 ± 0.59^{a}	7.6.±0.63	7.26±0.79
VDA	80±67	506.6 ± 79.43^{a}	$1361.\pm328.8^{ab}$	2.6 ± 0.82^{a}	6.46±1.12	7.93±0.70
SFB	55±24	502.6 ± 97.13^{a}	1424 ± 158.1^{b}	3.9 ± 0.87^{a}	5.93±0.70	7.46±0.83
TTJ	61±11	502.0 ± 116.38^{a}	1453 ± 316.0^{b}	3.5 ± 1.31^{a}	6.66±0.65	7.33±0.97
GMJ	54±08	503.3 ± 85.67^{a}	$1411.\pm269.4^{b}$	4.2 ± 0.90^{a}	6.26±0.79	7.06±0.79
TNM	55±18	502.3 ± 65.36^{a}	$1189.\pm102.9^{ab}$	3.7 ± 0.56^{a}	6.44±0.63	7.66±1.05
TMM	55±22	501.0 ± 75.76^{a}	$1239.\pm137.7^{ab}$	3.2 ± 0.56^{a}	6.59±0.81	7.81±0.72

ANFH: Average Nauplii at first hatch, FDL:First day length, TDL: Third day length, SDL: Sixth day length, LMSM: Length of male at sexual maturity, LFSM: Length of female at sexual maturity.

Artemia strain CKF: Kelambakam, Tamil Nadu; VDA: Vedaranyam, Tamil Nadu; TTJ: Tuticorin, Tamil Nadu; GMJ: Mithapur, Gujarat; TNM: Tamaraikulam, Tamil Nadu; TMM: Marakanam, Tamil Nadu and SFB: *Artemia franciscana*-San Francisco Bay strain.$^{\#}$ Superscript with different alphabets indicates the significant difference ($P<0.05$).

2.4.3 Cyst biometry

Cyst diameters exhibited considerable variation among the different geographical strains of indigenous *Artemia*. Cyst of the Marakanam strain (TMM) appeared to be the smallest with an average diameter of219.63 ± 10.99µm while that of Tamaraikkulam (TNM) appeared to be the largest with diameter of 236.37 ± 19.00µm. Among the exotic strains *A. tibetiana* (TBS) received from the *Artemia* reference center, Gent, Belgium possessed the largest cyst size (319.39 ± 24.74µm) followed by *A. salina*(261.72 ± 17.35 µm) while the commercial *A. franciscana* cyst size was closer to the Indian samples. One way ANOVA of the mean values of the cyst diameter did not shown any significant difference ($P>0.05$) among the indigenous strains and SFB except TNM. However, the cyst diameter of the indigenous strains and SFB were significantly different from that of TBS and ASL (Fig. 2. 7).

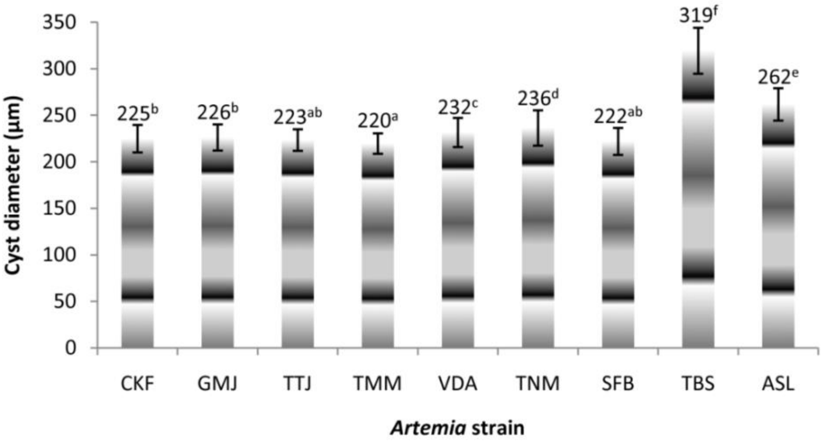

Fig 2.7 Mean cyst diameter (µm) of non-decapsulated cysts of indigenous and exotic *Artemia* populations with standard deviation and one way ANOVA. Superscripts with different alphabets indicates the significant difference between the *Artemia* populations (P<0.05).

2.4.4. Reproductive pattern

Individual culture experiments conducted for testing the breeding pattern of indigenous strains of *Artemia* from various hypersaline habitats of India and reference strain of *A. franciscana* revealed the total absence of parthenogenesis in any of them. All the *Artemia* individuals died after 3-4 months culture period without producing any nauplii/ cyst. The males and females reared together formed pre-copulatory riding pairs and reproduced sexually.

The diallel crossing of seven strains in forty nine combinations resulted in the formation of pre-copulatory riding pairs leading to the production of nauplii and cysts by all of them irrespective of the strains involved. Though the reproductive output showed variations, no infertile pairs were observed among the different crosses, indicating the absence of any reproductive barrier among the Indian populations as well as with the exotic A. *franciscana*. The entire crossbred progeny matured and produced F2 generation with different degrees of reproductive output, which further confirmed the fertility of the progeny. Different crossbred F2 progenies showed significant difference in first day length, while no significant differences were noted at the third day length, sixth day length and length at sexual maturity (P<0.05) (Table 2.3)

Table 2.3 Life history traits of the F1 generation cross bred *Artemia*

Strains in the cross		ANFH	FDL	TDL	SDL	LMS	LFS
Male	**Female**						
CKF	SFB	54±28	617.3±31.50^{ab}	1243.±244.1^{ab}	3.3±0.52^{a}	6.73±0.59	7.86±0.63
	VDA	32±6	597.3±40.08^{ab}	1108.±115.9^{ab}	2.8±0.25^{a}	6.20±0.75	6.46±1.12
	TTJ	75±29	541.3±55.40^{ab}	1246.±203.0^{ab}	2.8±0.65^{a}	6.86±0.83	7.86±0.83
	GMJ	79±10	596.0±35.81^{ab}	1296.±158.6^{ab}	4.6±0.60^{a}	7.00±0.8	7.73±0.45
	CKF	55±43	518.0±144.3^{a}	1153.±146.0^{ab}	3.5±0.59^{a}	7.6.±0.63	7.26±0.79
	TNM	63±13	533.3±35.40^{ab}	1206.±153.0^{ab}	2.6±0.45^{a}	6.44±0.54	7.45±0.76
	TMM	59±20	588.0±45.81^{ab}	1136.±144.6^{ab}	3.6±0.54^{a}	7.01±0.4	7.53±0.49
VDA	SFB	27±06	590.0±79.19^{ab}	1125±258.0^{ab}	2.9±0.99^{a}	7.06±1.48	9.06±1.33
	CKF	88±07	591.3±33.77^{ab}	1220±38.03^{ab}	1.2±0.26^{a}	6.8.±1.20	6.40±0.50
	TTJ	49±32	664.6±17.67^{b}	1383.±159.4^{ab}	3.5±0.51^{a}	7.33±0.61	7.73±0.88
	GMJ	46±14	576.0±55.78^{ab}	1266.±100.7^{ab}	3.3±0.71^{a}	6.66±0.89	7.33±0.61
	VDA	80±67	506.6±79.43^{a}	1361.±328.8^{ab}	2.6±0.82^{a}	6.46±1.12	7.93±0.70
	TNM	55±18	584.6±65.6^{ab}	1267.±109.4^{ab}	3.1±0.42^{a}	7.13±0.41	7.99±0.88
	TMM	67±21	596.0±65.78^{ab}	1254.±107.7^{ab}	3.2±0.66^{a}	6.77±0.69	7.29±0.61
SFB	CKF	56±25	603.3±30.86^{ab}	1473.±70.37^{b}	4.1±0.92^{a}	7.00±0.65	7.2±1.26
	VDA	98±25	557.3±90.03^{ab}	1328.±112.9^{ab}	3.1±0.54^{a}	6.20±0.77	7.73±1.38
	TTJ	61±21	588.6±49.26^{ab}	1403.±100.8^{b}	3.8±0.56^{a}	6.13±0.83	7.46±0.74
	GMJ	78±05	552.0±38.02^{ab}	1373.±148.0^{ab}	4.7±0.67^{a}	5.93±0.88	7.60±0.82
	SFB	55±24	502.6±97.13^{a}	1424±158.1^{b}	3.9±0.87^{a}	5.93±0.70	7.46±0.83
	TNM	72±23	537.3±70.03^{ab}	1301.±99.9^{ab}	3.3±0.44^{a}	6.50±0.77	7.66±1.18
	TMM	66±32	563.6±57.44^{ab}	1399.±111.8^{ab}	3.6±0.36^{a}	6.43±0.73	7.54±0.94
TTJ	CKF	37±09	614.6±71.30^{b}	1211.±076.7^{ab}	3.0±1.03^{a}	7.00±1.25	7.86±1.06
	VDA	23±08	591.3±49.98^{ab}	1096.±221.9^{a}	3.1±1.09^{a}	6.66±0.72	8.13±1.45
	SFB	44±01	591.3±70.08^{ab}	1323.±109.1^{ab}	4.1±0.77^{a}	6.60±0.63	7.93±0.70
	GMJ	23±08	580.0±33.38^{ab}	1271±146.0^{ab}	4.0±0.83^{a}	7.00±1.19	6.93±0.45
	TTJ	61±11	502.0±116.38^{a}	1453±316.0^{b}	3.5±1.31^{a}	6.66±0.65	7.33±0.97
	TNM	33±12	555.3±39.36^{ab}	1166.±111.9^{ab}	3.3±0.99^{a}	6.54±0.58	7.83±1.05
	TMM	43±13	543.3±50.12^{ab}	1356.±129.2^{ab}	4.0±0.43^{a}	6.34±0.92	7.90±0.50
GMJ	CKF	58±25	548.0±44.43^{ab}	1353.±69.35^{ab}	2.4±0.58^{a}	6.73±0.45	7.06±0.70
	VDA	51±08	506.0±48.37^{ab}	1121.±106.8^{ab}	2.7±0.59^{a}	6.60±0.72	7.40±0.91
	SFB	44±05	626.0±19.19^{b}	1000±67.48^{ab}	2.8±0.41^{a}	7.00±0.65	7.40±0.82
	TTJ	102±04	490.0±32.07^{a}	1175±227.3^{ab}	2.2±0.165^{a}	6.60±0.50	6.20±0.41
	GMJ	54±08	503.3±85.67^{a}	1411.±269.4^{b}	4.2±0.90^{a}	6.26±0.79	7.06±0.79
	TNM	59±11	566.0±59.19^{a}	1123±66.54^{ab}	2.6±0.81^{a}	6.56±0.75	7.30±0.61
	TMM	61±19	526.0±72.12^{ab}	1166±189.5^{ab}	2.6±0.43^{a}	6.69±0.33	6.89±0.87

TNM	CKF	37±12	565.6±62.34^{ab}	1191.±66.7^{ab}	3.1±1.13^{a}	6.99±1.05	7.92±1.06
	VDA	78±11	593.3±69.00^{ab}	1091.±154.3^{a}	3.3±1.01^{a}	7.00±0.82	8.23±1.35
	SFB	54±32	601.3±67.07^{ab}	1321.±145.6^{ab}	4.1±0.86^{a}	6.77±0.65	7.83±0.80
	TTJ	61±14	582.0±45.27^{ab}	1210±122.0^{ab}	3.6±0.52^{a}	6.78±1.11	6.99±0.65
	GMJ	44±14	532.0±96.38^{ab}	1403±299.0^{b}	3.3±1.03^{a}	6.43±0.43	7.29±0.77
	TNM	55±18	502.3±65.36^{a}	1189.±102.9^{ab}	3.7±0.56^{a}	6.44±0.63	7.66±1.05
	TMM	71±22	534.3±60.22^{ab}	1314.±159.2^{ab}	4.0±0.79^{a}	6.30±0.72	7.85±0.88
TMM	CKF	45±21	580.0±89.19^{ab}	1122±200.0^{ab}	2.6±0.89^{a}	7.00±1.33	9.02±1.53
	VDA	65±22	566.3±63.71^{ab}	1230±88.99^{ab}	1.9±0.46^{a}	6.71±1.29	6.9±0.90
	SFB	56±12	604.6±97.62^{ab}	1366.±150.49^{ab}	3.1±0.31^{a}	7.23±0.91	7.93±0.58
	TTJ	63±14	546.0±75.70^{ab}	1229.±99.76^{ab}	3.0±0.61^{a}	6.3±0.80	7.48±0.66
	GMJ	52±23	536.6±99.48^{ab}	1382.±228.8^{ab}	2.8±0.72^{a}	6.50±1.01	7.81±0.61
	TNM	62±23	541.6±85.60^{a}	1240.±119.4^{ab}	3.0±0.52^{a}	7.13±0.61	8.01±0.49
	TMM	55±22	501.0±75.76^{ab}	1239.±137.7^{ab}	3.2±0.56^{a}	6.59±0.81	7.81±0.72

ANFH: Average Nauplii at first hatch, FDL: First day length, TDL: Third day length, SDL: Sixth day length, LMSM: Length of male at sexual maturity, LFSM: Length of female at sexual maturity.

Artemia strain CKF: Kelambakam, Tamil Nadu; VDA: Vedaranyam, Tamil Nadu; TTJ: Tuticorin, Tamil Nadu; GMJ: Mithapur, Gujarat; TNM: Tamaraikulam, Tamil Nadu; TMM: Marakanam, Tamil Nadu and SFB: *Artemia franciscana*-San Francisco Bay strain.

[#] Superscript with different alphabets indicates the significant difference ($P<0.05$).

2.4.5. Molecular genetic characterization

Internally transcribed spacer region-1 (ITS-1) sequence data generated in the present study were deposited in the NCBI (Table 1I.4). The alignment of sequences generated (Table 2. 4) (partial sequence of 18S rRNA, ITS-1 complete sequence and 5.8S rRNA partial sequence) revealed a matrix of 1430 bp. The G+C content of the sequences ranged from 29.4 to18.4 with an average of 23.9. Size of the ITS-1 sequence was found to be ranging from 922 to 924 bp, matching with that of *A. franciscana* (923-924 bp) and showing 99% homology.

Table 2.4 Sample ID, Collection site and GenBank Accession number of ITS-1 sequence submissions from the present study

Individual ID	Collection site	Identified species	GenBank Accession No.
CKF 008	Kelambakam , Tamil Nadu, India	*A. franciscana*	GU323289
CKF 003			GU323317
GMJ 010	Mithapur , Gujarat, India	*A. franciscana*	GU323290
GMJ 006			GU323291
GNM 001	Nanganvadi , Gujarat, India	*A. franciscana*	GU323292
GNM 002			GU323293
GNM 003			GU323294
GNM 004			GU323295
GNM 005			GU323296
GNM 006			GU323297
SFB 004	San Francisco Bay, USA	*A. franciscana*	GU323298
SFB 005			GU323299
TMM 001	Marakkanam, Tamil Nadu, India	*A. franciscana*	GU323300
TMM 003			GU323301
TNM 001	Thamaraikulam, Nagercoil, India	*A. franciscana*	GU323302
TNM 002			GU323303
TNM 003			GU323304
TNM 004			GU323305
TNM 005			GU323306
TNM 006			GU323307
TTJ 002	Tuticorine, Tamil Nadu, India	*A. franciscana*	GU323308
TTJ 004			GU323309
TTJ 006			GU323310
TTJ 008			GU323311
TTJ 009			GU323312
VDA 003	Vedaranyam, Tamil Nadu, India	*A. franciscana*	GU323313
VDA 007			GU323314
VDA 010			GU323315
VDA 005			GU323316

Sequence analysis of ITS-1 revealed high polymorphism among the Indian *Artemia* populations. Twenty two haplotypes were observed out of the 29 *Artemia* individuals analyzed from the different populations (Table 11. 5). Haplotype A was found to be present in three strains viz, GNM from the Northwest coast, CKF & TTJ from Southeast coast of the Indian subcontinent (Table 2.6). Similarly, haplotype C was found to be present in two strains from distant geographical locations viz, VDA from Southeast region and GMJ from Northwest region. However, the haplotype B was restricted to strain from Southeast region only viz, TMM &TNM. The sequence characteristics of ITS1 are presented in Table 2.7.Out of the 16 polymorphic nucleotide sites, the parsimony informative sites were found to be few (5 only) in comparison with singleton variable sites (11). Nucleotide diversity and the population mutation parameter for ITS 1 region were found to be less in the Indian *Artemia* populations (Pi: 0.00283). The overall transition/transversion ratio of the ITS-1 sequences was found to be as R = 0.81. Tajima's D, based on the number of segregating sites (s) with the nucleotide diversity (π), was found to be -0.96775 (P<0.01). (Table 2.6)

Table 2.5 Haplotype diversity of the different *Artemia* strains

Haplotype	***Artemia* strain**								**Total**
	CKF	**TTJ**	**GMJ**	**GNM**	**SFB**	**TMM**	**TNM**	**VDA**	
A.	1	2	0	1	0	0	0	0	4
B.	0	0	1	0	0	0	0	0	1
C.	0	0	1	0	0	0	0	1	2
D.	0	0	0	1	0	0	0	0	1
E.	0	0	0	1	0	0	0	0	1
F.	0	0	0	1	0	0	0	0	1
G.	0	0	0	1	0	0	0	0	1
H.	0	0	0	1	0	0	0	0	1
I.	0	0	0	0	1	0	0	0	1
J.	0	0	0	0	1	0	0	0	1
K.	0	0	0	0	0	1	0	0	1
L.	0	0	0	0	0	1	0	0	1
M.	0	0	0	0	0	2	2	0	4
N.	0	0	0	0	0	0	1	0	1
O.	0	0	0	0	0	0	1	0	1
P.	0	1	0	0	0	0	0	0	1
Q.	0	1	0	0	0	0	0	0	1
R.	0	1	0	0	0	0	0	0	1
S.	0	0	0	0	0	0	0	1	1
T.	0	0	0	0	0	0	0	1	1
U.	0	0	0	0	0	0	0	1	1
V.	1	0	0	0	0	0	0	0	1

Table 2.6 Sequence characteristics of DNA segment comprising of Partial 18S rRNA, complete ITS1 partial 5.8S rRNA

Length range of the sequence (bp)	1048
Invariable (Monomorphic) sites (bp)	1018
G+C content range (mean)	29.4 ; 18.4 (23.9)
Singleton variable sites	11
Parsimony informative sites	5
Variable (polymorphic) sites	16
Total number of InDel sites analyzed	12
Average InDel event	1.091
Average InDel length	1.077
Tajima's D	-2.22161
Sequence conservation C	0.984

The pair-wise genetic distance between the Indian *Artemia* populations from different geographical locations appeared to be very low ranging from 0 to 0.01. The pair-wise genetic distance between the different native *Artemia* populations and also with the exotic *Artemia franciscana* ranged from 0 to 0.010 (mean, 0.003), indicating that all the native *Artemia* belong to the species *Artemia franciscana*. The mean pairwise genetic distances observed between the indigenous *Artemia* other *Artemia* species are given in Table 2.7. The mean genetic distance with that of *Artemia persimilis*, *Artemia salina*, *Artemia sinica*, *Artemiaurmiana*, *Artemia* species from China, *Artemia* species from Kazakisthan and *Artemia parthanogentica* were 0.536, 0.207, 0.1418, 0.140, 0.133, 0.130 and 0.130 respectively.

Table 2.7 Mean genetic distances of indigenous *Artemia* from other *Artemia* species.

Artemia Species	Mean genetic distance	Minimum	Maximum
A. franciscana	0.0030	0.000	0.010
A. salina	0.2074	0.180	0.228
A. sinica	0.1418	0.137	0.147
A. parthanogenetica	0.1300	0.126	0.135
A. persimilis	0.5364	0.530	0.554
A. china Sp.	0.1335	0.128	0.139
A. kazakisthan Sp.	0.1325	0.128	0.137
A. tibetiana	0.1300	0.126	0.135
A.urmiana	0.1406	0.136	0.145

Principal Coordinate Analysis (PCoA) clearly grouped the Indian *Artemia* populations and the reference *A. franciscana* in to a single cluster indicating its genetic similarity. The genetically distant *A. persimilis* (0.53 to 0.554) *A. salina* (0.18 to 0.228), *A. sinica* (0.1418 to 0.137) and *A. urmiana, A. tibetiana, A sp. China, A sp. Kazakisthan & A. parthenogenetica* (0.1300 to 0.1406) formed independent distinct clusters (Fig.2. 8).

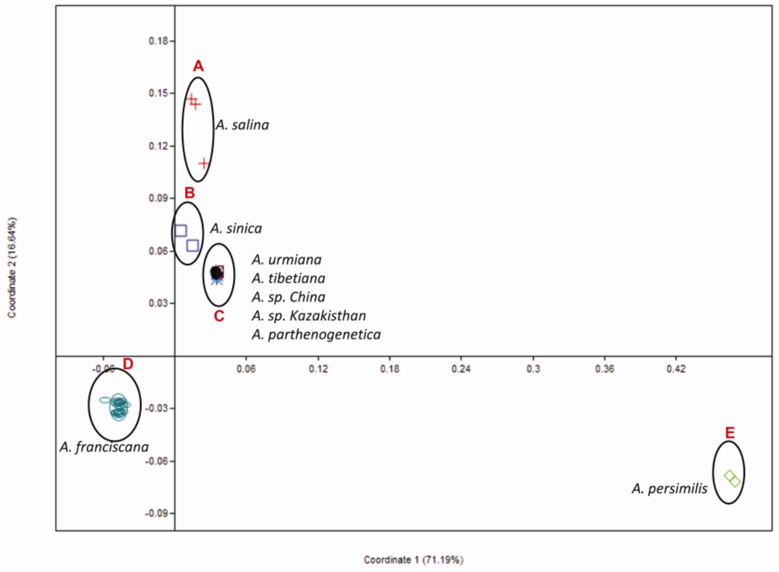

Fig.2. 8 Principal Coordinate Analysis (PCoA) of the different strains of *Artemia* species. PCoA was performed using the software PAST (Palaeontological Statistics) (Hammer *et al.* 2001).

Topologies of the phylogenetic trees constructed from the ITS1 sequences of different *Artemia* species using both Maximum Parsimony (MP) and Neighbour Joining (NJ) methods were found to be congruent, and hence the Maximum Parsimony tree is illustrated hereunder. Both trees revealed three main clades (A, B & C) with high bootstrap values (>79). In the MP tree, Indian *Artemia* populations and *A. franciscana* were grouped under a single clade A with a bootstrap value of 99. Clade B is subdivided into two, a unique *A. salina* group and a mixed group consisting of *A. sinica, A. urmiana, A. kazakhastan, A. tibetiana, A. china* and *A. parthenogenetica* with a bootstrap value of 79.The individuals of *A. persimilis* formed an independent clade C with a bootstrap value of 99 (Fig.2.9).

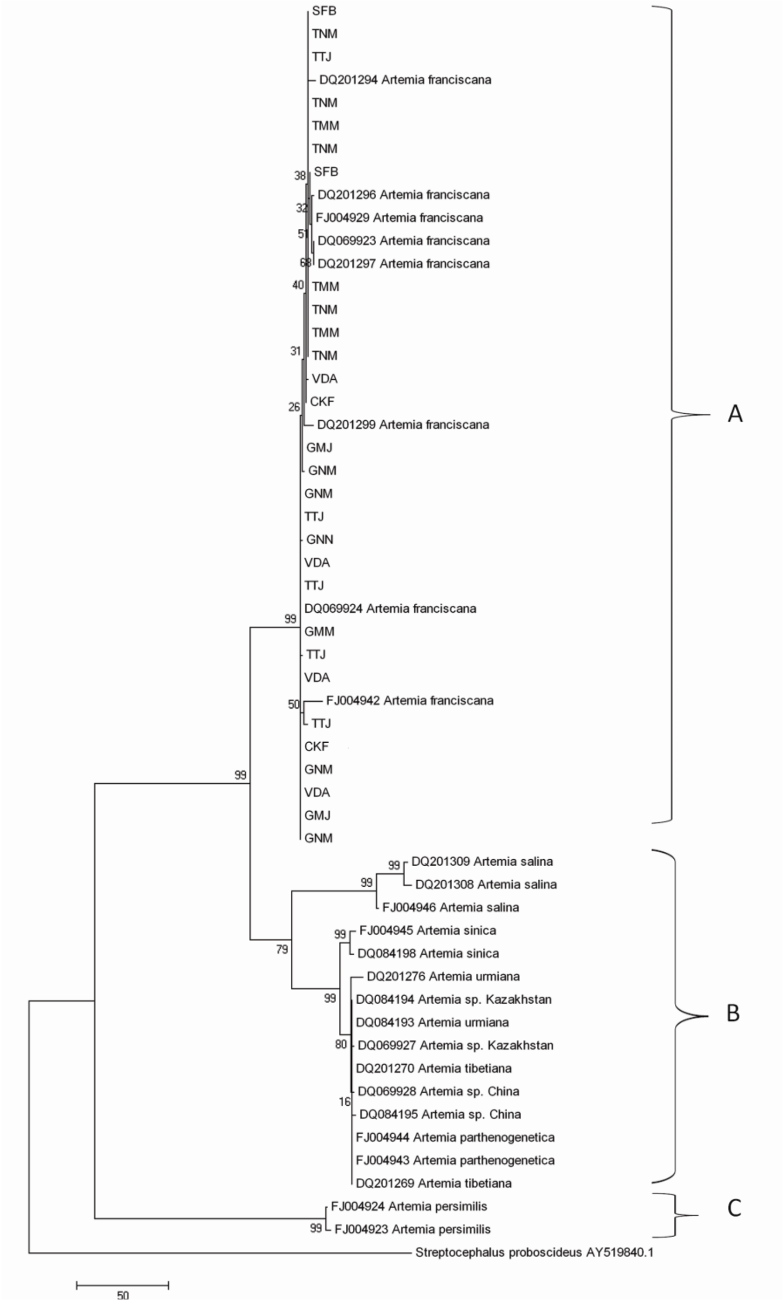

Fig.2.9 Phylogenetic tree of *Artemia* constructed by the maximum parsimony analysis using the Internal Transcribes Spacer 1 (ITS-1) region sequences of 29 samples from seven indigenous locations and 26 reference strains from the NCBI data base with 1000 bootstrapping. Where CKF: Kelambakkam, Tamil Nadu; VDA: Vedaranyam, Tamil Nadu; TTJ: Tuticorin, Tamil Nadu; GMJ: Mithapur, Gujarat; GNM: Nanganvadi, Gujarat; TNM: Thamaraikkulam, Tamil Nadu; TMM: Marakkanam, Tamil Nadu and SFB-*Artemia franciscana*: San Francisco Bay strain

2.5. Discussion

Although parthenogenetic *Artemia* populations have been reported from various geographical regions of India since 1950s, later studies revealed the gradual disappearance of *Artemia* populations from these regions. Parthenogentic *Artemia* populations were reported from both Sambhar and Didwana basins of the northwest region of India (Baid, 1958; Bhargava & Alam, 1980). However, Alam, (1980) reported the disappearance of *Artemia* from Sambhar during 1977-1978. Though Jakher *et al.*, (1990) observed *Artemia* populations in Didwana during 1984-1985, Jain *et al.*, (2005) reported the loss of *Artemia* fauna from this habitat based on his studies during 2000-2004, but failed to ascribe any reasons for its disappearance. The present study reveals that loss of *Artemia* has occurred from other habitats also. We couldn't find *Artemia* in Sambhar and Didwana lake of Rajasthan, and Vassai, Meera road, Bhayander, Nallasopara, Thane and Uran of Maharashtra where presence of parthenogenetic strains have been earlier reported.

The *Artemia* populations collected for this study during 2008-2010 were all sexually breeding / bisexual, composed of males and females which were fertile. Assessment of the reproductive pattern clearly showed that none of the individually reared *Artemia* could produce any offspring parthenogenetically confirming the total absence of parthenogenesis. On the contrary, the breeding experiments clearly showed that all these populations were able to sexually breed, producing fully fertile offspring, thus exhibiting their breeding efficiency and fertility. Absence of any infertile pairs among the different crosses indicated the absence of any reproductive barrier among the Indian populations as well as with the exotic *A. franciscana*, as reported by Sugumar, (2010). The variations exhibited in the reproductive output and cyst/nauplii numbers are quite normal and the entire F2 progeny matured which confirmed their fertility.

The hatching percentage of the cysts is an important criterion that determines the quality of *Artemia*. High hatching percentage (HP) along with short incubation periods and synchronous hatching ensures uniform sized nauplii with high nutrient quality and reduces periodical harvesting and wastage, thereby enhancing the profitability in hatchery operations. Abatzopoulos *et al.,* (2006) have reported hatching rates varying from 13 to 97% in *Artemia urmiana* strains, and stated that variations in HRs of wild collected *Artemia* cysts are a common phenomenon. John *et al.* (2005) have observed an HR of 69.2% in the stored cysts of *Artemia parthenogenetica* from Tamaraikulam (TMM) while the HP for the bisexual *Artemia* strain from the same region observed during the present study was 52.4%. The HPs observed in the Indian strains during the present study ranged from 45% (TTJ) to 84.5% (GMJ) while in the commercial SFB strain it was 72.2%, indicating the better quality of the GMJ strain.

It should be noted that the cyst diameters of the Indian *Artemia* from different geographical locations did not show any significant difference except for the TMM strain from Marakanam. The cyst size of Indian strains of *Artemia* were closer to the exotic *A. franciscana* strain than to the native *A. parthenogenetica* earlier reported by John, (2004), and other exotic bisexual *Artemia* (248 to 286µm) (Abatzopoulos *et al.,* 2006; Kuruppu and Ekaratne, 1995; Kara *et al.*, 2004). With respect to the cyst size, the Marakkanam strain (TMM) appeared to be the smallest (219.0µm) while the Tamaraikulam strain (TNM) had the largest cyst (236.4µm). The difference in cyst size among the strains appeared consistent during the entire two year (2008 to 2010) sampling period and hence the size of the cysts can be used as a preliminary tool to differentiate various *Artemia* populations as have been suggested by Moraiti-Ioannidou *et al.,* 2007) and Abatzopoulos *et al.*,1988). These findings also point out to the possible spread of *Artemia franciscana*, which have been imported and introduced to India as live feed for Aquaculture since the early eighties, in the Indian salterns. In a similar study, widespread distribution of *A.*

franciscana in the Western Mediterranean region was observed by Amat*et al.*, (2007) based on adult *Artemia* biometry.

Nauplii length (NL) of the various indigenous *Artemia* strains showed marginal difference, and ranged from 496.0 to 514.8µm (Fig.2.10). Marakkanam strain (TMM) possessed smallest nauplii (496µm).Nauplii length of the native strains are comparable with that reported for *Artemia franciscana* (487.1 to 490.7µm) by Shirdhankar *et al.*, (2004), Greek *Artemia* sp. (455.0 to 507.4 µm) by Moraiti-Ioannidou *et al.*, (2007), *Artemia urmiana* (466.3 to 505.0µm) by Abatzopoulos *et al.*, (2006), Italian Margherita di Savoia *Artemia* (517.0µm) by Vanhaecke and Sorgeloos, (1980) and Portugal Samouco *Artemia* (503.5µm) by Vilela *et al.*, (1987). However, Indigenous *Artemia* possessed significantly smaller nauplii when compared to *Artemia tibetiana* (667.0µm) strain (Abatzopoulos *et al.*, (1998).

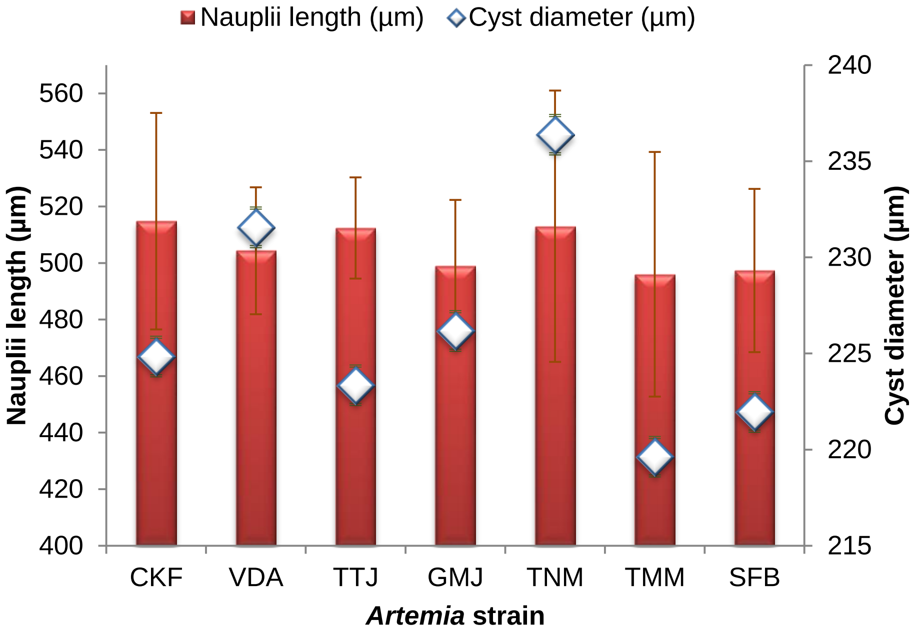

Fig. 2.10 Nauplii length and Cyst diameter of different *Artemia* strains. Primary vertical axis (Y) represents the average Nauplii length (µm) with standard deviation and secondary vertical axis (Z) represents the average Cyst diameter (µm) with standard deviation.

The small mouth opening in most finfish and shellfish impose mechanical restriction on the capture and ingestion of the food particle. Hence, any small size starter diet with optimum nutrient level raises interest. Therefore, small sized nauplii have a vital role in the feeding of the hatchery reared larvae. The present study have shown that, size wise, nauplii of the indigenous *Artemia* strains are comparable to /or even better than the most widely used commercial strain, and so is suitable for use as candidate live feed. Other life history estimates like TDL, SDL, LMSM and LFSM values, though, showed significant differences were comparable with other *Artemia* species but do not have much relevance in the present context.

Confirmatory proof for the extensive presence of exotic, sexually breeding *Artemia franciscana* populations in the Indian hypersaline habitats has come from the molecular studies carried out under this programme. Earlier studies in crustaceans have shown that though the ITS-1 is highly variable across species, it is highly conserved within the species (Chu *et al.*, 2001; Von der Schulenburg *et al.*, 2001) and hence can be used as an excellent molecular tool for molecular taxonomy. Comparison of the ITS-1 sequences of the indigenous populations with other *Artemia* species in this study have shown that the Indian population(s) were very close to the exotic *A. franciscana* with 99% homology, while other *Artemia* species such as *A.* sinica, *A. urmiana, A. tibetiana, A. salina, A. persimilis* and *A. parthenogenetica* are distant.

The mean pair-wise genetic distances (GD) within the Indian Artemia populations were found to be negligible (0 to 0.01), indicating that all the Indian populations is genetically similar and can be considered as a single species. The absence of any significant difference in the GD (0 to 0.01) between the different Indian *Artemia* populations and *A. franciscana* indicate again their conspecific nature. On the other hand the GD between the Indian populations and other *Artemia* species *viz. A. persimilis, A. salina, A. sinica, A. urmiana, Artemia* China sp., *A. Kazakhstan* sp. and *A. parthenogenetica* were substantially high, indicating that they belongs to a different species. Nucleotide diversity (Nei & Tajima, 1981) is one of the most commonly used indexes for estimating the genetic diversity of a population when DNA sequence data for individuals in that population are available. In the present study nucleotide diversity in the Indian *Artemia* populations (Pi: 0.00283) was observed to be very negligible indicating low genetic diversity.

Phylogenetic analysis also confirmed that the present day Indian *Artemia* belongs to *A. franciscana* species. While the Principal coordinate Analysis (PCoA) clearly grouped the Indian *Artemia* populations and the reference *A. franciscana* in to a single cluster and the remaining genetically distant species viz, *A. persimilis, A. salina* and *A. sinica* have differentiated into another clade, the old world *A. persimilis* stood alone separately.

The topologies of Maximum Parsimony (MP) and Neighbour Joining (NJ) trees were found to be very similar, indicating that they support each other. Four main clades with high bootstrap values (>79) were clearly distinguishable in phylogenetic tree. The indigenous strains and the reference *Artemia franciscana* formed one clade with a low range of bootstrap values (>63). The topologies of the phylogenetic tree revealed the close relationship of the indigenous *Artemia* strains with the exotic *Artemia franciscana* which could be distinguished from other *Artemia* species. The lower bootstrap value within the main clad is possibly because all of them belong to the same species and there is low intra specific variations. Higher value for other clad may be explained by the reason that they belong to different species and the inter-specific variations are higher than intra-species variations. These results showed that the sequence variation in ITS-1 region of *Artemia* is a very effective tool for phylogenetic study at the species level.

Our findings are in agreement with the observations of Barigozzi, (1989), that *A. persimilis* is a primitive species, from which *A. franciscana*, *A. sinica* and *A. urmiana* originated at different time periods of evolution. *A. persimilis* being older, shares traits with old world species like *A. salina*, whilst *A. franciscana* which is younger, is thought to be under evolutionary expansion due to its great colonizing ability (Gajardo *et al.*, 2002). Clade B consisted of 2 groups, a unique *A. salina* and a mixed group consisting of *A. sinica*, *A. urmiana*, *A. kazakhastan*, *A. tibetiana*, *A. china* and *A. parthenogenetica.* Within this mixed group, our observations indicate a close relationship between *A. parthenogenetica*, *A. tibetiana*, *A. china* strain, *A. Kazakhstan* and *A. urmiana* strain which is in agreement with the previous reports postulating that the diploid parthenogenetic *Artemia* lineages are closely related to *A. urmiana* (Baxevanis *et al.*, 2006) and *A. Kazakhstan* is closely related to *A. parthenogenetica* group (Muñoz *et al.*, 2008). This was evident from the PCoA, where *A. parthenogenetica*, *A. tibetiana*, *A. china* strain, *A. Kazakhstan* and *A. urmiana* formed a single cluster, distinct from *A. franciscana* and the Indian *Artemia* samples, *A. sinica*, *A. salina* and *A. persimilis* cluster (Fig.2.11).

Kappas *et al.*, (2004) has observed that the peculiar biotic nature of the *Artemia* habitats may lead to the development of endemic groups of *Artemia* in later stages of evolution. Present investigation reports the lack of regional endemism in different Indian *Artemia* populations in distant habitats like Northeast, Southeast and Northwest regions of the Indian subcontinent indicating that these populations are comparatively young in the process of evolution. Haplotype diversity throws light into the distribution pattern of the Indian *Artemia* populations. Haplotype diversity studies have shown 22 haplotypes out of 29 *Artemia* individuals from geographically distant habitats. Haplotype A was present in three distinct habitats in the Northwest and Southeast regions, haplotype C was observed in the Southeast and Northwest regions, while haplotype B was restricted only to the Southeast region of the country (Fig.2.11).

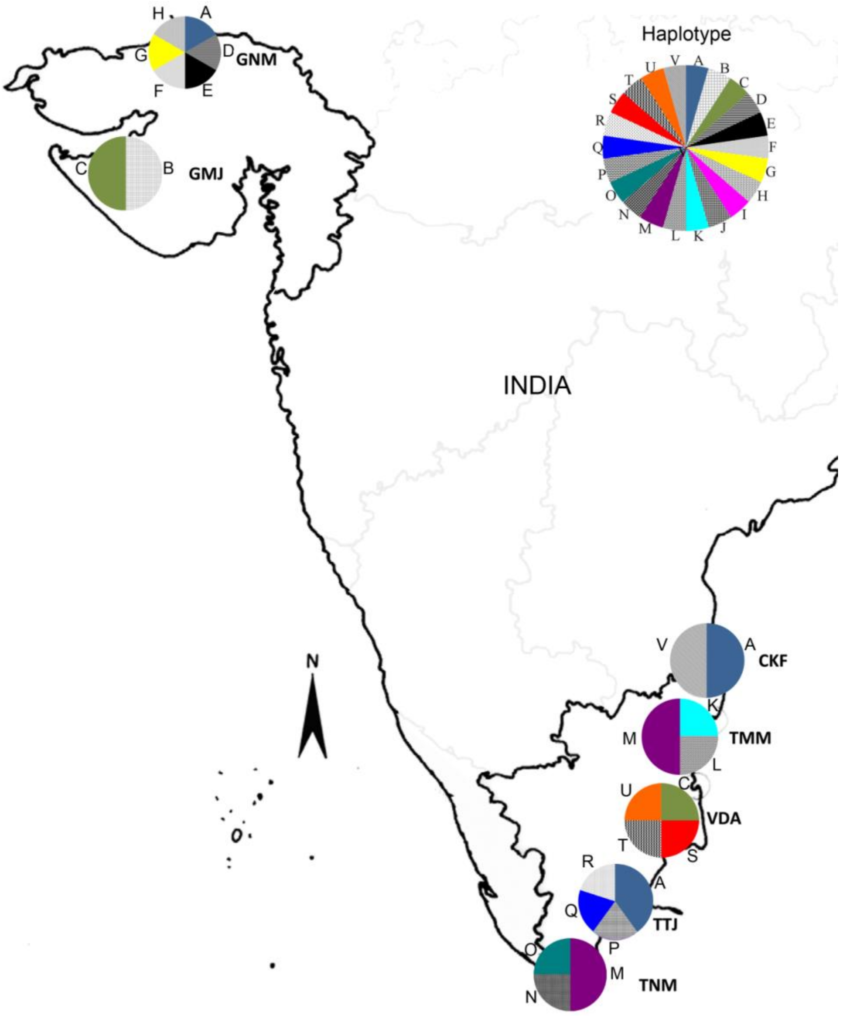

Fig.2. 11 Haplotype map of the different *Artemia* strains collected from various hypersaline habitats of India. Twenty two haplotypes (A to V) were assigned individual colors, where CKF: Kelambakkam, Tamil Nadu; VDA: Vedaranyam, Tamil Nadu; TTJ: Tuticorin, Tamil Nadu; GMJ: Mithapur, Gujarat; GNM: Nanganvadi, Gujarat; TNM: Thamaraikkulam, Tamil Nadu and TMM: Marakkanam, Tamil Nadu.

The distribution of same haplotypes in geographically distinct habitats indicates that they have a common origin but are evolving cohesively (Parkin & Butlin, 2004). High singleton variation in the ITS-1 sequence as revealed by the sequence characteristics (Table 2.4), is a key indicator that the population is under expansion while the presence of few parsimony informative sites indicate the relative absence of 'bottle necks' in the expansion of this population. The negative Tajima's D value, higher singleton variations, and less number of parsimony informative sites in the ITS-1 sequence of the Indian *Artemia* populations signify an excess of low frequency polymorphisms, which again indicate that the population is under expansion and/or positive selection (Tajima, 1989). The transitions to transversions ratio (TI:TV ratio) estimated from the aligned nucleotide sequences showed more frequent transversions than the transitions (R = 0.81), which provokes the high frequent transition bias (DeSalle *et al.*, 1987).

The morphological and biometric observations, crossbreeding results, molecular data, especially the very high ITS-1 sequence homology, negligible genetic distance and phylogenetic analysis clearly proves that the *Artemia* populations collected from the various geographical regions of India are *A. franciscana,* naturalized during the course of time. These observations indicate that *A. parthenogenetica* (Kulkarni, 1953; Baid, 1968; Royan, 1970 & 1979; Achari, 1971; Bhargava & Alam, 1980; Basil *et al.*, 1987; Kulasekharapandian *et al.*, 1992; John, 1994) have been replaced by sexually breeding *A. franciscana* that have invaded and naturalized. In a similar study, colonization induced extinction of local populations by *A. franciscana* has been reported from Mediterranean habitats by Amat, (2005; 2007).

A. franciscana, have been exported worldwide since the 1950s for use as live feed in hatcheries. The massive import of *A. franciscana* as live feed following the phenomenal growth of the Indian aquaculture industry since the early eighties has paved the way for its entry and establishment in the Indian ecosystems. Species extinction is an ecological process happening in nature and displacement by exotic species is one of the reasons (Cheng *et al.*, 2009). Occurrence of species or populations in non-native environments reveals their ability to migrate/invade and establish along with native populations and in course of time naturalize in the new habitat. High degree of phenotypic plasticity helps the invasive species to enter and expand in new habitats in the face of hostile environmental conditions (Browne & Wanigasekera, 2000; Clegg *et al.*, 2000; Tanguay*et al.*, 2004). Generally, sexually breeding populations have high dispersal capacities and competitive advantage over the asexual species, which leads to invasion and establishment (Wilson, 1992). According to Amat *et al.,* (1991) *A. franciscana* as an invasive species around the world as they compete with the native brine shrimp populations due to their superior adaptive capabilities. Various physical agents such as wind and water; biological carriers like migratory aquatic birds and predators also might have contributed to the invasion of this exotic species across the different saline habitats of India.

The disappearance of parthenogenetic *Artemia* populations the North West region of India could be ascribed to the extreme draught conditions in these habitats (Rao, 2009). In the east coast of the Indian subcontinent, the states of Andhra Pradesh, Orissa and West Bengal have numerous aquaculture farms and hatcheries where *Artemia* has been extensively used as live feed. But surprisingly, our study revealed total absence of *Artemia* in all the hypersaline water bodies of the region. This could be due to the climatic conditions of the region where these hypersaline habitats are prone to frequent cyclone associated heavy rainfall and floods annually (Khatua & Dash, 2000), preventing the establishment of *Artemia* sp.. Whereas the North West (Gujarat) and South East (Tamil Nadu) regions have the most suitable climatic conditions and most of the hypersaline areas are naturally protected from flooding, helping the populations to tide over any adverse conditions and flourishes. Numerous hatcheries situated in the areas must have paved way for the invasion of *A. franciscana* populations into the nearby hypersaline habitats. Moreover, these locations fall in the migratory route of flamingoes and this may also have helped in the dispersion of the species, which is in agreement with the views expressed by Green *et al.,* (2005).

In the light of the finding of a massive invasion of *A. franciscana* into the natural hypersaline habitats in the Indian subcontinent where it has flourished and displaced the native *A. parthenogenetica* from its natural habitat, the species-ecosystem and habitat interaction has to be validated further to shed more light on this aspect. This forms the first report on the loss of the native *A. parthenogenetica* from the Indian hypersaline water bodies following the invasion of by *A. franciscana,* a species already known for invasions worldwide. For assessing the changes in the flora and fauna of the regions concerned brought about by such invasions require further studies.

2.6. Conclusion

Morphometric and molecular analysis of the indigenous *Artemia* from different geographical location were carried out for their characterization and species identification. The present investigation sheds light on the massive invasion and naturalization of the exotic *Artemia franciscana* species into the Indian saline ecosystems, displacing the native parthenogenetic *Artemia*. Their cyst size and nauplii size were comparable with the commercial strain of *A. franciscana* and even better in some of the geographic strains. The exceptional phenotypic plasticity, broad tolerance to diverse environments and the breeding pattern of the alien *A .franciscana* species might have facilitated its invasion in to the Indian subcontinent. This forms the first report on the displacement of parthenogenetic *Artemia* by the invading *A. franciscana*. Further studies are required to understand the changes brought by such invasions in the particular ecosystems.

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Chapter 3

Evaluation of nutrient composition of *Artemia* strains collected from Indian Salinas

Vikas P.A, Kajal Chakraborty, Sajeshkumar N.K, Thomas P.C, Sanil, N.K, Vijayan K.K. (2013).
Quality evaluation of six allochthonous Artemia franciscana strains from the hypersaline habitats of Indian subcontinent. ***Journal of Applied Aquaculture***. Accepted

3.1. Abstract

Sustainable larval production is vital for the success of aquaculture and the production of quality larvae requires starter diet with superior nutritional qualities. Live feeds are vital in delivering the essential nutrients required for the growth and development of finfish and shell fish larvae in hatcheries. Compared to artificial diets, live feeds are always better in terms of acceptability, texture and nutrient profile. Though different stages of *Artemia* have been widely used as live feed, the nutritional superiority combined with the smaller size makes the nauplii stage, the most suitable. Though *Artemia* are widely distributed in various hyper-saline habitats of the Indian coast, they are not being used as starter diet in larviculture. Instead, all the requirements are met through the imported exotic strains. Hence, need was felt to identify indigenously available strains and evaluate their nutrient quality for use as live feeds for Indian aquaculture.

In the present study six strains of naturalized *Artemia franciscana* from different habitats of India and one commercial strain (Great Salt Lake origin *Artemia franciscana*) were evaluated for their nutrient profile. The biochemical constituents of nutritional importance estimated were soluble protein, indispensable amino acids (IAA), dispensable amino acids (DAA), carotenoids, polyunsaturated fatty acids (PUFAs) and trehalose. Soluble protein was less in the cyst stage but increased in the nauplii and adult stages. Higher levels of indispensable amino acids were observed in the nauplii stage as compared to the cyst and adult stages. Adult stages had higher carotenoid content, while Tamaraikulam (TNM) and Marakkanam (TMM) strains showed the highest values when compared to the SFB strain. Pearson analysis showed a positive correlation between the carotenoid and protein contents in the different stages of *Artemia* ($P<0.05$). Trehalose content of the cyst and hatching percentage showed a significant positive correlation indicating the role of trehalose in keeping the viability of the cyst. Poly unsaturated fatty acid was higher in the nauplii as compared to the adult and cyst stages. TMM and GMJ possessed the highest 20:5n-3 and 22:6n-3 content and appeared to be superior to the other strains with respect to the nutrient indicators studied. Such indigenous strains with higher nutritional profile can be used as live feed through large scale farming, as base generation for development of superior strains or for further genetic improvement through selective breeding.

Keywords: *Amino acid; Carotenoid; Fatty acid; Protein; Trehalose*.

3.2. Introduction and review of literature

Sustainable larval production is vital for the success of aquaculture. Non availability of healthy, uniform sized, quality seeds in sufficient quantities have always been a concern in '*capture based aquaculture'* where seeds are sourced from the wild. The above issues can be addressed through hatchery production of quality seeds in sufficient quantities. The larviculture operations in the hatchery are controlled, and hence, the specific requirements for the developing larvae like selected live/artificial diets, optimum conditions etc. can be provided. The use of hatchery developed seeds shall also relieve the pressure on the wild caught seeds. All these have forced the aqua-industry to move towards '*hatchery based aquaculture'* systems (Sadovy de Mitcheson and Liu, 2008).

Once the exogenous feeding starts, the growth rate of the larvae depends mainly on the availability of suitable, nutrient-rich starter diet (Rønnestad *et al.,* 1999). Live feeds play vital role in delivering the essential nutrients required for the growth and development of finfish and shell fish larvae in hatcheries (Narciso *et al*., 1999).Many live feeds, especially *Artemia* nauplii, possess an edge over the formulated diets due to their higher acceptability, texture and nutrient profile. Several hypotheses have been proposed to explain higher acceptability of *Artemia* nauplii by the predatory larvae which specifically points out its peculiarities in biochemical composition, structure and digestibility of its dietary protein, exogenous enzymes, chemical and visual stimuli (Garcia – Ortega *et al*., 1998).

Due to the high growth rate in the early developmental phase of the larvae, the starter diet should hold high levels of soluble proteins, amino acids, carotenoids and fatty acids, especially the *n*-3 and *n*-6 polyunsaturated fatty acids (PUFA). The nutritional value of *Artemia* as live feed is mainly determined by of n-3 highly unsaturated fatty acids (HUFA). Watanabe *et al.,* (1978) have grouped *Artemia* into two groups: freshwater- type, with n-3 unsaturated fatty acids such as linolenic acid (18:3n-3) but lacking the eicosapentaenoic acid (20:5n-3), producing good survival and growth among freshwater animals, and the marine-type whose lipids contain 20:5n-3 thereby making them suitable as feeds for marine animals. Specifically, variations in the fatty acid composition of *Artemia* cysts and nauplii are determined by the differences in the fatty acid composition of the food ingested by the parental population and also due to their genetic makeup (Ruiz *et al*., 2008).

It is well known that in finfish and shell fish larvae, the *n*-3 and *n*-6 PUFAs play a major role in many physiological processes (Clegg and Trotman, 2002; Dantagnan *et al.,* 2010). They have important roles in membrane structure and function and also serve as an important energy sources during the early development of the larvae (Tocher, 2003). Fatty acid composition of *Artemia* varies among strains, and hence estimation among all available strains is important.

Carotenoids act as photoprotectors and appear to play an important role in immunological functions, antioxidant defense mechanisms and alleviation of oxidative stress in larvae (Kranner and Birtic, 2005; Alonso-Alvarez *et al.,* 2007; Peters, 2007). Wouters *et al.,* (2001) have reported that carotenoids play vital roles in the larval pigmentation, scavenging of free radicals, and protection of eggs from oxidative deterioration and prevention of peroxidation of PUFA in the diet, which should be supplied externally through diets to impart these qualities in the larvae. Moren *et al.,* (2004) have reported that in *Artemia* nauplii, β-carotene is the dominant carotenoid, but in the adults canthaxanthin commands a larger share, indicating the depletion of β-carotene in them. The concentration of canthaxanthin and β-echineone, which are the β-oxidized products of the precursor molecule (*β*-carotene) are not abundant in the cysts. On the contrary, these carotenoids have been reported to be abundantly available in the nauplii and the adult stages.

Apart from these nutrients, amino acids (AA) are also very important during the early growth phase of the larvae. Since the early growth phase is characterized by muscle protein deposition, a high flow of amino acids (AA) is required for the growing biomass (Conceição *et al.,* 1998; Rønnestad *et al.*, 2003). Further, AAs are also used by the larvae as an immediate source of energy which in turn increases the dietary AA demand (Wright and Fyhn, 2001).The ideal level of dietary requirement of AAs in starter diets is based on the absorption efficiency of the individual AAs, the AA profile of proteins being synthesized and on the preferential use of individual AAs for energy or other purposes (Conceição *et al.*, 2003). Several AAs such as arginine, alanine, phenylalanine, lysine, aspartic acid and glycine are known to stimulate feeding in the growing larvae (Aragão, 2004). Hence, knowledge about the AA profile and their composition in the starter diet is essentially required to manage the initial larval feeding.

Trehalose is a protective solute, preserving membranes from desiccation, and proteins from thermal denaturation and aggregation (Clegg and Conte, 1980; Crow *et al.*, 1998; Godelieve and Macrae, 2002). Trehalose synthesis in Diapause-destined embryos begins at about two days post fertilization and its level may vary with the development stage and the strain. Hence trehalose may have a vital role in deciding the hatching percentage of the *Artemia*. Further, these essential nutrients play a vital role in prompting the survivability and development of the larvae (Chakraborty *et al.*, 2010; Abatzopoulos *et al.*, 2006).

Artemia of all life stages such as the cyst and the nauplii are used as potential live feed in larviculture (Sorgeloos *et al.,* 2001) but compared to the nauplii others are less preferred. Adult *Artemia* biomass can be used as a feed ingredient for crustacean larvae since it provide additional benefits such as induction and reinforcement of sexual maturation, and for increased fertilization rates (Naessens *et al.,* 1997; Wouters *et al.,* 1997). According to Malpica Sanchez *et al.,* (2004) the bioencapsulation technique provides interesting opportunities for using *Artemia* biomass not only as a live feed, but also as a carrier for administration of various products to the predator, such as essential nutrients, pigments, hormones, and prophylactic or therapeutic agents.

Artemia cysts consist of dormant *Artemia* embryos covered with a three-layered cyst shell. Under optimal hatching conditions, the embryos break out of the shell and hatch into nauplii, which are used for feeding the larvae or fry of species with a high commercial value (Lim *et al.*, 2002). According to Garcia – Ortega *et al.,* (1998) the cysts of *Artemia* present some advantages to artificial diets because they combine the physical properties of a dry artificial feed and the nutritional value of live *Artemia* nauplii. An important application of the decapsulated cysts is the direct feeding to the predator, in which case, even low-hatch or no-hatch cysts can be used for feeding (Lim *et al*., 2002).

During the cyst hatching process, the pre-nauplius larva, surrounded by its hatching membrane, protrudes from the empty shell, floating as a result of its buoyancy, giving rise to the appearance of floating umbrella, and is called as umbrella-stage *Artemia*. Nhu *et al.,* (2009) detailed the ability of cobia larvae to ingest and digest the umbrella-stage *Artemia* at first feeding as well as it’s nutritional suitability as a starter diet replacing the rotifers.

Though different *Artemia* strains are found widely distributed in various hyper-saline habitats across the globe (Stappen, 2002),based on their nutrient quality, naupliar size and hatching efficiency, only few strains are used extensively as starter diet in larviculture. Therefore, identification or development of strains with higher qualities shall be of great significance in aquaculture.

Recent studies on the *Artemia* strains form Indian hyper-saline habitats using morphological examination and breeding pattern have revealed the displacement of *A. parthenogenetica* with sexually breeding strain. Breeding experiments and molecular studies of these strains confirmed that the present Indian strains are naturalized *Artemia franciscana* (Vikas *et al.*, 2012).In India, *Artemia* has been used as live feed in the hatcheries since the early eighties and presently the aquaculture industry consumes approximately 2000 tone of *Artemia* annually, which has been met through imports (CMFRI, 2006). But, so far no studies have been carried out to evaluate the quality and suitability of indigenous *Artemia* strains as candidate live feed for Indian aquaculture. Therefore, evaluation of their nutrient profile is of utmost importance. In this background, the present study was carried out to evaluate the indigenous *Artemia* strains from various geographical locations with respect to their soluble protein, indispensable amino acids (IAA), dispensable amino acids (DAA), carotenoids, trehalose and fatty acids, so as to identify strains with high nutritional content. Such germplasm can be used as potential *Artemia* sources in larviculture industry and can reduce the import expenditure to the country.

3.3. Materials and methods

An extensive survey for the collection of indigenous strains of *Artemia* was conducted in the salinas of the coastal states of Indian sub-continent. Since

Artemia were available only in the hyper-saline habitats of Mithapurin the Northwest region, Kelambakam, Vedaranyam, Tamaraikulam, Marakkanam and Tuticorinin the Southeast region of India, they were collected from those locations and designated as GMJ, CKF, VDA, TMM, TNM, and TTJ respectively (Fig.3.1). The *Artemia* cysts collected from these habitats were packed in polyethylene bags before being brought to the laboratory. The cysts were cleaned and processed by biphase floatation technique with brine and fresh water and stored at 4^0C until further use. Great Salt Lake commercial strain *Artemia franciscana* (SFB) (Ocean Star International, Snowville, USA) was used as the reference strain in this study.

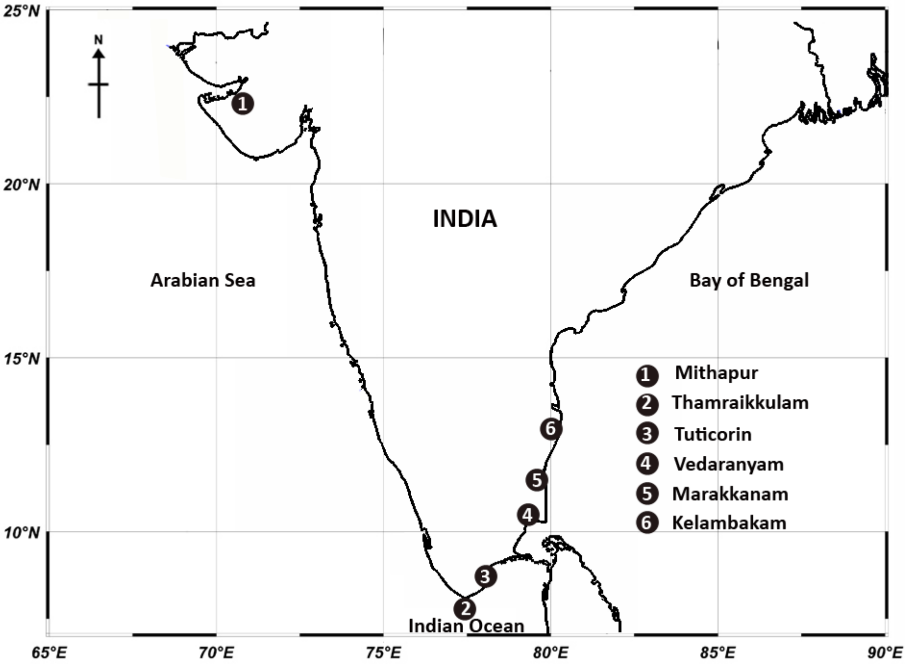


Fig. 3.1 Schematic map showing the geographical locations of *Artemia* in Indian subcontinent

The geographical coordinates of collection sites.Mithapur: 22^0 24’ N, 69^0 01’E, Thamaraikkulam 10^0 51’ N, 72^0 02’E, Tuticorin: $08^0$46’ N, 78^0 08’ E, Vedaranyam: 10^0 22’ N, 79^0 51’ E, Marakkanam 12^0 10’ N, 79^0 54’E and Kelambakam: $12^0$47’ N, 80^0 13’ E.

3.3.1. Biochemical analyses of different stages of *Artemia*

3.3.1.1. Chemicals and instrumentation

The solvents used for the sample preparation were of analytical grade (E-Merck, Darmstadt, Germany). Gas Liquid Chromatography (GLC) data were recorded on a Perkin-Elmer Auto System XL gas chromatograph. Standards of fatty acid methyl ester (Supelco TM 37 component FAME mix, catalog no. 47885-U) and boron trifluoride/methanol (14% BF_3 in methanol, w/v) were procured from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). All glassware's were rinsed with $CHCl_3$-CH_3OH (2: l v/v), and dried under N_2. The GC analyses were accomplished on a Perkin Elmer Gas chromatograph equipped with an Supelco wax capillary column (Supelco, Belfonite, USA, 100 m X 0.25 mm i.d.) using a flame ionization detector equipped with a split/splitless injector, which was used in the split (1:15) mode. The oven temperature ramp program: 80°C for 10 min, rising at 5°C min-^{1} to 280 °C; injector and detector temperatures were 280 °C; carrier gas N_2 (ultra-high purity >99.99%, 25 mL/min). The injection port temperature was maintained at 280 °C. The injection volume was 0.01µL.

The soluble proteins, carotenoids, trehalose, Amino acids, and fatty acid contents of the six indigenous strains and one reference strain were estimated. For this, representative samples of cysts, freshly hatched nauplii and laboratory-cultured adults from all these strains were lyophilized (Martin Christ Alpha-1-4 LD freeze-drier, Osterode, Germany) and preserved till analysis.

3.3.1.2. Estimation of soluble protein

Soluble protein content of the cyst, nauplii and adult *Artemia* were estimated using standard method with suitable modifications (Lowry *et al.*, 1951). Bovine serum albumin (BSA, 4 mg/mL) in distilled water was used as standard for the estimation. The absorbance of the protein aliquot was measured at 660 nm in an ultraviolet/visible (UV) spectrophotometer (Cary 50, Varian, USA) against reagent blank. The protein content of the sample was calculated from the standard curve of BSA, and expressed in percentage.

3.3.1.3. Estimation of total carotenoids

Total carotenoid content of the cyst, nauplii and adult samples were determined by standard methods with suitable modifications (Tolasa *et al.*, 2005; Olson, 1979). In brief, the samples (100 mg) were ground with anhydrous Na_2SO_4 (500mg) and the contents were dissolved in $CHCl_3$ (1 mL). The homogenates were incubated overnight at 0^oC and clarified by centrifugation (10,000 rpm for 20 min). The clarified $CHCl_3$ layer (0.4 mL) was diluted (10 times) with ethanol and scanned using spectrophotometer (Cary 50, Varian, USA) through λ=200 to 800. The samples were then quantified by reading the absorbance at 475 nm. Concentration of carotenoid was calculated from the formula: total carotenoid (µg / gm) = (absorbance X 10)/ (0.25 X sample weight (gm)), dilution factor = 10, extinction coefficient = 0.25. The carotenoid extracts were further qualitatively characterized by thin layer chromatography (TLC) using $CHCl_3$/MeOH (9:1 v/v) and the spots were visualized using UV and visible light. The identities were confirmed by comparing the results with reference standards.

3.3.1.4. Estimation of amino acids

Amino acid content of the cyst, nauplii and adults were estimated following the Pico-Tag method (Heinrikson and Meredith, 1984) with suitable modifications. In brief, *Artemia* samples (0.1-0.05g) with HCl (6N, 10mL) were digested at 110°C in sealed glass tubes for 24h on a multi-place heating mantle. The samples were allowed to cool and filtered through the GF/A grade glass fiber filter paper to obtain the filtrate, which was flash evaporated thrice, using distilled water (20 mL X 3) to remove the acid. The acid-free sample was then made up to 5 mL with HCl (0.05 N), and filtered through a nylon filter syringe (0.2 µ). The aliquot containing hydrolyzed amino acids were treated with re-drying reagent (methanol 95%: water: triethylamine 2:2:1 v/v/v), and thereafter pre-column derivatization of hydrolysable amino acids was performed with phenylisothiocyanate (PITC, or Edman's reagent) to form phenylthiocabamyl (PTC) amino acids. The reagent was freshly prepared, and the composition of derivatizing reagent comprises methanol 95%: triethylamine: phenylisothiocyanate (20µL, 7:1: l v/v/v, 70 µL methanol + 10 µL distilled water + 10 µL triethylamine +10 µL phenyl isothiocyanate). The contents were thoroughly mixed using a vortex mixer and allowed to stand at room temperature for 20 min, and the entire reagent was thereafter removed under vacuum. The derivatized sample (PTC derivative, 20 µL) was diluted with sample diluent (20 µL, 5 mM sodium phosphate NaHPO4 buffer, pH 7.4: acetonitrile 95:5 v/v) before being injected into the reversed-phase binary gradient high performance liquid chromatography (HPLC, Waters reversed-phase PICO.TAG amino acid analysis system), fitted with a packed column (dimethylocatadecylsilyl- bonded amorphous silica; Nova-Pak C18, 3.9 X 150 mm) maintained at 38±1°C in a column oven to be detected by their UV absorbance (λmax 254 nm). The mobile phase consists of eluents A and B, where eluent A comprises of sodium acetate trihydrate (0.14 M, 940 mL, pH 6.4) containing triethylamine (0.05%), mixed with acetonitrile (60 mL), and eluent B was acetonitrile : water (60:40, v/v). A standard gradient elution programme, recommended by Waters with increasing eluent B was employed for this purpose, and reported here (Table 3.1). An additional step of 100% eluent B is used to wash the column prior to returning to initial conditions. The detector (Waters 2487 dual absorbance detector) was set at 0.1 absorbance units full scale (AUFS) at 254 nm, and the column temperature was set at 38°C (±1°C) in the column heater. Standard (PIERS amino acid standard H) was run before each sample injection. Samples (PTC amino acid derivatives) were injected in triplicate, and the output was analyzed using BREEZE software. The quantification of amino acids was carried out by comparing the sample with the standard, and the results were expressed as triplicates with mean ± SD.

Table 3.1 HPLC gradient elution programme for the determination of amino acids

Time (min)	Flow(mL/min)	Percentage of eluent A	Percentage of eluent B
0.0	1.0	100	0
10.0	1.0	54	46
10.5	1.0	0	100
11.5	1.0	0	100
12.0	1.5	0	100
12.5	1.5	100	0
20.0	1.5	100	0
20.5	1.5	100	0

Reversed-phase binary gradient high performance liquid chromatography (HPLC, Waters reversed-phase PICO.TAG amino acid analysis system), fitted with a packed column (dimethylocatadecylsilyl bonded amorphous silica; Nova-Pak C18, 3.9 X 150 mm maintained at 38±1°C in a column oven) was used to analyze hydrolysable amino acids by their UV absorbance (λmax 254 nm). The mobile phase eluents used were A and B, where eluent A comprised of sodium acetate trihydrate (MeCOONa, 0.14 M, 940 mL, pH 6.4) containing triethylamine (Me3N, 0.05%), mixed with acetonitrile (60 mL); and eluent B used was acetonitrile : water (60:40, v/v).

3.3.1.5. Estimation of trehalose

Trehalose content of the *Artemia* cyst, nauplii and adult were estimated following the standard methods with suitable modifications (Carpenter and Hand, 1986; Jermyn, 1975). *Artemia* samples (50 mg) were homogenized in 6% perchloric acid (2ml) and the supernatant neutralized by saturated sodium carbonate and the perchloric salts were removed. The homogenates were made up to10 ml with distilled water, and from each homogenate 0.5 and 1.0 ml samples were taken along with the working standards (0.2, 0.4, 0.6 and 0.8 ml of glucose), and were made up to 1 ml with ice cold distilled water. To this, 4 ml of Anthrone reagent was added and digested for 8 minutes at 100 °C in a water bath and values were estimated at 630 nm in a spectrophotometer (Cary 50, Varian, USA). Trehalose content in samples was calculated from the graph using the following equation. Amount of Trehalose in the Sample = [(Sample value from the graph (mg)/ (Volume of sample)) X (Total Volume of extract (ml) / (Weight of sample in (mg))] X 100.

3.3.1.6. Estimation of fatty acids

Total lipid content of the cyst, nauplii and adult samples were extracted by the method reported by Bligh and Dyer (1959) with suitable modifications. In brief, the samples (500 mg) were extracted with $CHCl_3$/MeOH (60 mL, 2:1, v/v) and water (20 mL). The $CHCl_3$ layer was processed to recover the triglycerides which were saponified with alkaline reagent (3 mL, 0.5 N KOH/MeOH). The saponified materials were thereafter reacted with a methylating mixture (14% BF_3/CH_3OH) to yield methyl esters (FAME) that were subsequently extracted with *n*-hexane/H_2O (1:2, v/v) (Metcalfe *et al.*, 1966). After the removal of the aqueous layer, the *n*-hexane layer was passed through Na_2SO_4, concentrated in vacuum, reconstituted in petroleum ether and stored at -20°C until further analysis. The composition of the fatty acids was analyzed using a Perkin Elmer Auto System XL, Gas chromatograph (Perkin Elmer, USA) equipped with a flame ionization detector.

3.3.2. Statistical analyses

Descriptive statistics were computed for all the traits and means were examined for significance by one way analysis of variance (ANOVA). Pearson correlation coefficient of the trehalose content of the cyst with hatching percentage was estimated ($P<0.05$).All the statistical analyses were carried out using the software package SPSS 13.0 (SPSS Inc, Chicago, USA).

3.4. Results

3.4.1. Nutrient evaluation

3.4.1.1. Soluble protein content

Soluble protein content in the cyst, nauplii and adult stages of indigenous *Artemia* varied significantly (Table 3.2). Among the different life cycle stages, adult (38.6 ± 6.0%) and nauplii (35.6 ± 8.2%) showed significantly higher soluble protein levels as compared to the cyst (11.7 ± 2.3%). Among the various strains, cysts of GMJ (16.3%) and CKF (13.1%) showed significantly higher protein levels than the other strains (9.7 to 10.8%) and the reference strain (11.3%). A major incremental trend in soluble protein content was observed in the nauplii stage as compared to the cyst stage in all the strains. It was high in the nauplii of TTJ (48.1%) and CKF (45.5%) strains when compared to the reference SFB (36.6%) and other indigenous strains. In case of adults, the CKF and SFB strains showed significantly higher soluble protein content (48.3 & 42.4%) than VDA (38.0%), GMJ (36.2%) and TTJ (28.8%) strains ($P<0.05$) studied.

3.4.1.2. Carotenoid content

In all the strains evaluated, the carotenoid content was high in adult (754.9 µg/gm) than the nauplii (266.9 µg/gm) and cyst stages (259.5 µg/gm) (Table 3.2). Adults of the TMM and TNM strains showed significantly higher carotenoid content (1317.4 & 1082.2 µg/gm) followed by CKF (863.6 µg/gm) and TTJ strains (831.6 µg/gm). In the nauplii, higher carotenoid was observed in TMM, TNM and GMJ strains (403.6, 326.8 & 310.1 µg/gm) while in others it ranged from 172 to 293 µg/gm. Among the cysts, higher carotenoid was observed in TNM and TMM strains (408.9 & 390.2 µg/gm) when compared to the reference SFB strain (126.4 µg/gm) and other strains.

Thin layer chromatography (TLC) of the crude carotenoid fraction revealed the presence of β-carotene at R_f: 0.85 in cysts. Canthaxanthin (R_f: 0.7) and β-echineone (R_f: 0.75), and other β-oxidized products of β-carotene were not apparent in cysts. However, these carotenoids were found to be present in the nauplii and the adult stages. The adult populations were found to possess carotenoids of higher polarity, *viz.*, phoenicoxanthin (R_f: 0.50), asthxanthin (R_f: 0.45), and its esteranalogues (R_f: 0.25-0.40). The presence of polar carotenids (β-echineone and canthaxanthin) in thenaupliiar stage was also supported by the hyperchromic shift of λ_{max} from cyst (ε=9000, 474 nm) to nauplii (ε=12000, 477 nm).

3.4.1.3. Trehalose content

Trehalose content was significantly high in the cyst stage (8.9%) than in the nauplii (5.7%) and adult stages (3.9%) of the seven *Artemia* strains evaluated (Table 3.2). In cysts, trehalose content ranged from 5.6 to 11.5 per cent with maximum levels in GMJ (11.5%) followed by SFB (10.1%) and TMM (10.02%) strains. After transition from the cyst to nauplii, trehalose level was significantly low, and it ranged from 4.1 to 7.6 percent. The trehalose content showed a further decrease in the adult stage (3.3 to 4.8%) irrespective of the strains. Pearson correlation analysis revealed a positive correlation between the hatching percentage and trehalose content of *Artemia* cyst ($P<0.05$).

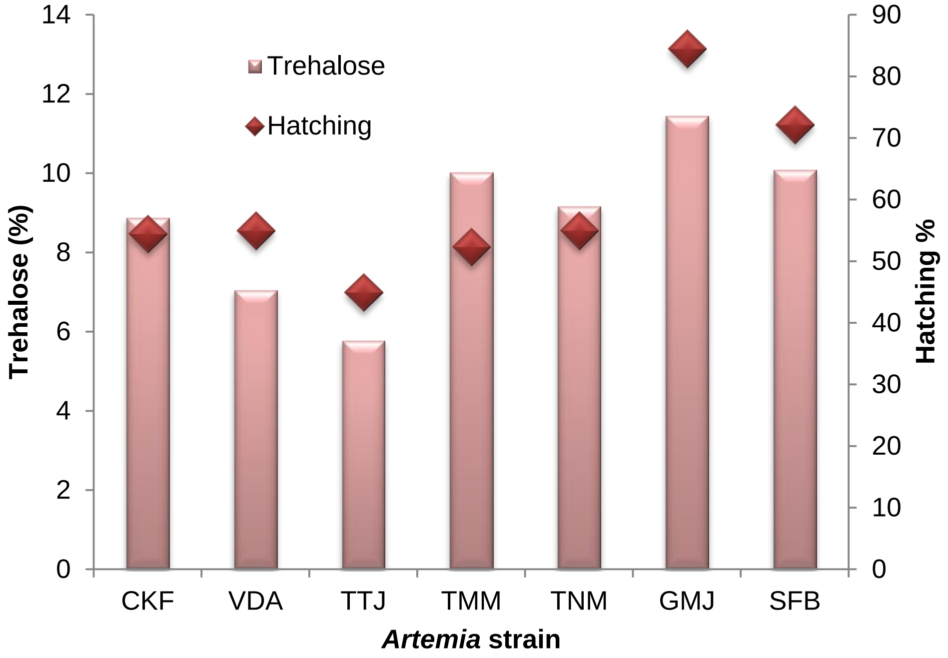


Fig.3.2. Trehalose content of Cyst and hatching percentage of the various *Artemia* strains

CKF: Kelambakam, VDA: Vedaranyam, TTJ: Tuticorin, TMM: Marakanam, TNM: Tamaraikulam, GMJ: Mithapur- Gujrat and SFB: *A. franciscana* strain.

Table 3.2 Protein, carotenoid and trehalose content of life cycle stages of indigenous *Artemia* strains.

***Artemia* Strain**	**Protein (%)**[1]	**Carotenoid (µg/gm)**[2]	**Trehalose (%)**[3]
		Cyst	
CKF	13.10 ± 0.09^{a}	203.03 ± 0.19^{a}	8.88 ± 0.19^{d}
VDA	10.75 ± 0.25^{b}	122.25 ± 2.51^{b}	7.06 ± 0.16^{c}
TTJ	10.25 ± 0.25^{b}	238.60 ± 0.37^{d}	5.78 ± 0.11^{a}
GMJ	16.30 ± 0.19^{c}	327.09 ± 0.56^{c}	11.46 ± 0.21^{f}
TNM	09.65 ± 0.34^{d}	408.87 ± 0.03^{f}	9.17 ± 0.19^{b}
TMM	10.50 ± 0.50^{b}	390.22 ± 0.29^{f}	10.02 ± 0.18^{d}
SFB	11.25 ± 0.25^{b}	126.43 ± 0.37^{b}	10.09 ± 0.21^{e}
Avg.	11.68 ± 2.3	259.50 ± 125.0	8.92 ± 1.90
		Nauplii	
CKF	45.50 ± 0.50^{a}	174.12 ± 0.03^{a}	7.40 ± 0.11^{d}
VDA	29.10 ± 0.09^{b}	186.91 ± 0.10^{b}	5.83 ± 0.12^{c}
TTJ	48.10 ± 0.40^{c}	293.96 ± 0.06^{c}	4.06 ± 0.10^{a}
GMJ	32.25 ± 0.25^{d}	310.12 ± 0.11^{d}	4.96 ± 0.11^{b}
TNM	27.25 ± 0.25^{g}	326.76 ± 2.75^{d}	4.64 ± 0.11^{e}
TMM	30.50 ± 0.50^{f}	403.60 ± 0.56^{f}	7.58 ± 0.18^{b}
SFB	36.60 ± 0.40^{e}	172.95 ± 0.19^{a}	6.87 ± 0.15^{d}
Avg.	35.61 ± 8.2	266.91 ± 123.6	5.65 ± 1.30
		Adult	
CKF	48.38 ± 0.49^{a}	863.55 ± 0.52^{a}	4.82 ± 0.13^{c}
VDA	38.00 ± 0.50^{b}	439.09 ± 0.42^{b}	3.52 ± 0.14^{a}
GMJ	36.15 ± 0.54^{c}	447.07 ± 0.28^{b}	4.23 ± 0.15^{b}
TTJ	28.75 ± 0.44^{e}	831.57 ± 114^{a}	3.72 ± 0.11^{a}
TNM	40.08 ± 0.57^{e}	1082.2 ± 0.89^{d}	3.42 ± 0.15^{b}
TMM	36.33 ± 0.55^{c}	1317.4 ± 0.63^{e}	3.33 ± 0.14^{a}
SFB	42.40 ± 0.40^{d}	303.21 ± 0.32^{c}	4.13 ± 0.18^{a}
Avg.	38.58 ± 6.0	754.87 ± 374.1	3.88 ± 0.50

All values are mean ± SD; SD; standard deviation. Column wise vales with same letter (a, b, c, d, e, f) superscript are not significantly different ($P<0.05$).

[1] The true protein of *Artemia* cyst, nauplii and adult estimated by Lowry's method with modification (Lowry *et al*, 1951). *Artemia* samples were homogenized in 10%TCA and the OD was measured in 660 nm against the blank. Bovine serum albumin (4 mg/mL) has been used as standard.

[2] Total carotenoids was analyzed in *Artemia* cyst, nauplii and adult by using the method as described by Tolasa *et al.*, (2005) and Olson, (1979). The homogenized *Artemia* samples incubated at 0 ^{0}C overnight with chloroform. Quantification of total carotenoid was done by reading the optical density at 380, 450, 475 and 500nm in a spectrophotometer.

[3] Trehalose content of the sample were estimated following the standard method with suitable modifications (Carpenter and Hand, 1986; Jermyn, 1975). *Artemia* samples were homogenized in Perchloric acid and neutralized by saturated sodium carbonate and removed perchloric salts. Anthrone reagents were added to the samples and digested in a water bath and read absorbance at 630 nm. Trehalose content in *Artemia* samples were calculated from the results.

3.4.1.4. Amino acid content

Cyst: Indispensable amino acid (IAA) in the cysts of different *Artemia* strains ranged from 917.7 to 1507.6 mg/100gm (Table 3. 3). Among the strains, IAA was highest in SFB (1507.6 mg/100gm) followed by VDA (1212.8 mg/100gm) and TTJ (1152.4 mg/100gm). Lysine, leucin and valine contributed the major share of the total IAA (226.4, 180.6 and 151.3 mg/100gm respectively), while histidine and methionine appeared to be very low (57.0 and 63.6 mg/100gm). Other IAAs viz, isoluecine, tyrosine, phenylalanine, threonine and valine ranged between 101.0 to 203.7 mg/100gm.

The levels of dispensable amino acids (DAA) of cysts were higher than IAA, and ranged from 1316.9 to 2223.2 mg/100gm. DAA was the highest in VDA strain (3197.5 mg/100gm) followed by the reference SFB (2223.2 mg/100gm) and other strains. Highest IAA/DAA ratio was observed in the TMM strain (0.45) followed by GMJ (0.43) and TNM strains (0.42), while it was low in CKF (0.35), VDA (0.38) and SFB (0.40). Among the DAAs, glutamine, arginine and aspartic acid showed significantly higher values (382.9, 282.4 and 266.3 mg/100gm respectively) compared to others. Cystine contributed the least in the total DAA, and ranged from 10.8 to 27.7 mg/100gm.

Table 3.3 Amino acid content of the *Artemia* cyst

Amino acid (mg/100gm)	CKF	VDA	TTJ	GMJ	TNM	TMM	SFB	Avg.
Ile	69.51 ± 1.0	112.84 ± 1.9	129.67 ± 1.9	125.77 ± 1.9	119.39 ± 2.0	119.8 ± 1.3	172.53 ± 1.4	121.36
Leu	173.55 ± 1.5	192.31 ± 2.4	174.88 ± 2	166.98 ± 2.0	153.26 ± 1.0	155.5 ± 2.4	247.94 ± 2.1	180.63
Tyr	58.13 ± 0.5	120.06 ± 1.8	111.42 ± 2	100.43 ± 1.8	149.47 ± 2.1	127.9 ± 2.6	109.73 ± 1.9	111.02
Met	62.85 ± 0.7	65.37 ± 1.1	58.33 ± 0.8	66.67 ± 0.9	54.02 ± 1.3	51.1 ± 0.4	86.62 ± 0.9	63.57
Phe	101.84 ± 1.4	123.84 ± 1.9	112.65 ± 1.3	109.74 ± 1.7	120.12 ± 1.3	111.5 ± 2.0	154.34 ± 1.1	119.15
Thr	111.6 ± 1.6	132.68 ± 1.2	124.08 ± 1.8	99.92 ± 1.4	90.04 ± 0.9	83.1 ± 1.0	169.56 ± 1.9	115.85
Val	83.57 ± 0.8	150.99 ± 2	166.54 ± 2.0	150.61 ± 1.9	152.62 ± 2.1	151.1 ± 1.7	203.67 ± 2.6	151.30
His	53.43 ± 0.4	65.87 ± 1.8	49.31 ± 0.8	54.19 ± 0.6	57.99 ± 0.4	57.8 ± 0.6	60.1 ± 0.9	56.96
Lys	203.22 ± 2.3	248.81 ± 3	225.54 ± 2.1	224.04 ± 2.6	167.31 ± 1.9	212.4 ± 2.4	303.12 ± 4.2	226.35
IAA	917.70	1212.77	1152.42	1098.35	1064.22	1070.20	1507.61	1146.18
Asp	214.98 ± 2.5	307.95 ± 3.3	285.8 ± 3.0	267.16 ± 3.0	207.3 ± 2.7	204.4 ± 3.0	376.54 ± 3.9	266.30
Glu	406.16 ± 3.5	452.1 ± 4.5	360.4 ± 4.2	351.41 ± 3.7	279.52 ± 3.4	275.6 ± 2.4	555.22 ± 6.4	382.92
Ser	173.76 ± 1.2	247.96 ± 3.1	238.28 ± 3.6	183.21 ± 2.0	195 ± 2.4	170.2 ± 2.1	282.75 ± 3.6	213.02
Gly	238.45 ± 2.5	194 ± 2.4	122.5 ± 1.9	104.82 ± 1.4	129.47 ± 1.8	105.3 ± 1.2	181.57 ± 2.0	153.73
Arg	395 ± 3.0	345.23 ± 4.1	246.59 ± 3.5	219.63 ± 2.8	191.29 ± 2.3	178.7 ± 1.9	400.38 ± 3.9	282.40
Ala	172.07 ± 1.5	242.27 ± 2.9	205.88 ± 2.7	172.41 ± 1.9	205.19 ± 1.8	180.4 ± 2	232.66 ± 3.0	201.55
Pro	101.21 ± 1.1	171.16 ± 1.9	167.42 ± 1.9	146.77 ± 1.8	222.54 ± 2.3	174.9 ± 1.3	172.67 ± 2.4	165.24
Cys	27.65 ± 0.2	24.09 ± 0.8	12.31 ± 0.2	10.84 ± 0.2	19.68 ± 0.3	27.4 ± 0.7	21.42 ± 0.8	20.48
DAA	**1729.28**	**1984.76**	**1639.18**	**1456.25**	**1449.99**	**1316.90**	**2223.21**	**1685.65**
Total	**2646.98**	**3197.53**	**2791.60**	**2554.60**	**2514.21**	**2387.10**	**3730.82**	**2831.83**
IAA/DAA	0.35	0.38	0.41	0.43	0.42	0.45	0.40	0.40

All values are mean ± SD; SD = standard deviation.

Ile: Isoleucine, Leu: Leucine, Tyr: Tyrosine, Met: Methionine, Phe: Phenylalanine, Thr: Threonine, Val: Valine, His: Histidine, Lys: Lysine, IAA: Indispensable amino acid, Asp: Aspartic acid, Glu: Glutamine, Ser: Serine, Gly: Glycine, Arg: Arginine, Ala: Alanine, Pro: Proline, Cys: Cystine and DAA: Dispensable amino acid

Nauplii: High IAA was observed in the nauplii of TTJ strain followed by TMM strain (2138.9 and 2081.1 mg/100gm) which were found to be significantly higher than the reference SFB strain (1310.2 mg/100gm). IAA values in other strains ranged from 1408.3 to 1997.6 mg/100gm (Table 3.4). Lysine, leucine, threonine and valine was high (335.0, 295.4, 264.9 and 231.5 mg/100gm respectively) as compared to tyrosine and methionine (55.8 and 77.8 mg/100gm). Total amino acid content was significantly high in TMM, TNM and TTJ strain nauplii (5524.71, 5524.7 and 5462.5 mg/100gm respectively) than the reference strain SFB (3365.8 mg/100gm) and other indigenous strains (3892.5 to 4856.4 mg/100gm). The IAA/DAA ratio in nauplii were low in all the strains (0.36 to 0.39). Among the DAAs, proline, glycine and alanine were low in the nauplii stage (160.0, 161.3 and 195.2 mg/100gm) when compared to the other amino acids such as glutamine, arginine and aspartic acid (651.7, 432.9 and 471.3 mg/100gm) (Table 3.4). Total DAA content values was high in TNM, TMM and TTJ strain nauplii (3527.07, 3436.79and 3323.59 mg/100gm respectively) than the reference strain SFB (2055.52 mg/100gm) and other indigenous strains (2484.23 to 3059.9 mg/100gm).

Table 3.4 Amino acid content of the *Artemia* nauplii

Amino acid (mg/100gm)	CKF	VDA	TTJ	GMJ	TNM	TMM	SFB	Avg.
Ile	195.49 ± 2.5	155.51 ± 1.6	240.83 ± 3.2	158.28 ± 2.3	218.83 ± 3.4	233.06 ± 3.1	148.62 ± 2.2	192.95
Leu	302.75 ± 3.7	236.84 ± 2.5	362.52 ± 4.2	245.36 ± 3.1	334.12 ± 4.1	357.83 ± 2.7	228.55 ± 1.8	295.42
Tyr	52.62 ± 0.7	41.56 ± 0.3	71.29 ± 0.6	43.8 ± 0.3	62.74 ± 0.6	81.17 ± 0.9	37.38 ± 0.6	55.79
Met	88.72 ± 0.9	61.33 ± 0.8	106.41 ± 1.3	66.84 ± 0.6	94.52 ± 1.2	89.98 ± 0.4	36.83 ± 0.7	77.80
Phe	188.08 ± 2.1	148.39 ± 1.9	236.88 ± 3	159.47 ± 1.9	209.03 ± 2.9	242.14 ± 2.7	138.7 ± 1.4	188.96
Thr	281.26 ± 3.5	217.56 ± 2.3	332.15 ± 2.9	235.05 ± 2.8	305.83 ± 4.1	286.26 ± 3.2	196.23 ± 2.5	264.91
Val	240.59 ± 2.9	192.04 ± 2.1	287.46 ± 2.1	199.97 ± 2.1	271.67 ± 3.1	256.02 ± 2.9	172.79 ± 1.9	231.51
His	103.28 ± 1.5	81.32 ± 0.9	101.78 ± 1.9	84.22 ± 0.1	115.05 ± 1.9	145.03 ± 1.4	72.63 ± 0.8	100.47
Lys	343.73 ± 4.8	273.73 ± 3.1	399.54 ± 3.2	274.09 ± 3.1	385.85 ± 2.4	389.62 ± 3.6	278.5 ± 3.2	335.01
IAA	**1796.52**	**1408.28**	**2138.86**	**1467.08**	**1997.64**	**2081.11**	**1310.23**	**1742.82**
Asp	525.89 ± 6.3	440.28 ± 5.3	489.13 ± 4.1	427.72 ± 4.5	576.67 ± 4.2	492.96 ± 5.1	346.18 ± 4.2	471.26
Glu	665.96 ± 4.9	562.71 ± 3.9	760.09 ± 5.3	559.88 ± 3.9	792.68 ± 3.9	723.26 ± 4.7	497.28 ± 5.3	651.69
Ser	378.9 ± 3.0	294.58 ± 2.9	443.85 ± 3.2	313.94 ± 2.9	425.95 ± 2.3	325.28 ± 3.3	233.94 ± 3.2	345.21
Gly	157.45 ± 2.1	123.39 ± 1.3	203.05 ± 1.9	139.99 ± 2	171.48 ± 1.9	218.27 ± 2.1	115.36 ± 1.7	161.28
Arg	429.32 ± 3.6	330.08 ± 4.2	511.32 ± 4.1	352.9 ± 3.5	465.34 ± 3.9	584.41 ± 5.3	356.55 ± 4.2	432.85
Ala	205.17 ± 2.8	171.51 ± 2.1	226.81 ± 2.9	172.72 ± 1.3	243.38 ± 2.3	212.32 ± 2.1	134.2 ± 1.4	195.16
Pro	163.68 ± 1.3	134.37 ± 1.5	191.25 ± 1.2	144.84 ± 1.9	183.67 ± 1.3	189.96 ± 1.9	112.08 ± 1.9	159.98
Cys	533.54 ± 5.5	427.31 ± 4.9	498.09 ± 3.3	458.42 ± 4.1	667.9 ± 5.2	690.33 ± 5.2	259.93 ± 3.1	505.07
DAA	**3059.9**	**2484.23**	**3323.59**	**2570.41**	**3527.07**	**3436.79**	**2055.52**	**2922.50**
Total	**4856.43**	**3892.51**	**5462.45**	**4037.49**	**5524.71**	**5517.90**	**3365.75**	**4665.32**
IAA/DAA	0.37	0.36	0.39	0.36	0.36	0.38	0.39	0.37

All values are mean ± SD; SD; standard deviation.

Ile: Isoleucine, Leu: Leucine, Tyr: Tyrosine, Met: Methionine, Phe: Phenylalanine, Thr: Threonine, Val: Valine, His: Histidine, Lys: Lysine, IAA: Indispensable amino acid, Asp: Aspartic acid, Glu: Glutamine, Ser: Serine, Gly: Glycine, Arg: Arginine, Ala: Alanine, Pro: Proline, Cys: Cystine and DAA: Dispensable amino acid

Adult: Lowest IAA was evident in adults (578.7 mg/100gm) when compared to cyst and nauplii stages. Except lysine (111.1 mg/100gm) and luecine (96.2 mg/100gm), all other amino acids were found to be low in the adults (Table 3.5) with methionine showing the least (17.6 mg/100gm) value. Among the strains, IAA values were observed to be high in TTJ and TNM (612.7 and 602.2 mg/100gm).

The DAA levels in the adults (795.1 mg/100gm) were found to be significantly lower than in cysts and nauplii of the various strains studied. Significantly higher DAA was observed in TTJ, TNM and VDA strains (850.3, 822.1 and 821.3 mg/100gm) than in the reference (764.0 mg/ 100gm) and other indigenous strains evaluated (746.6 to 791.0 mg/100gm). Cystine was significantly low in the adults (2.8 mg/100gm) than all the other DAAs estimated. Major share of DAAs were contributed by glutamine followed by arginine and aspartic acid (194.8, 188.5 and 124.0 mg/100gm). Total amino acid was high in the TTJ and TNM strains (1463.0 and 1424.3 mg/100gm) than that of the reference strain (1354.3 mg/100gm). The IAA/DAA ratio in adults ranged from 0.41 to 0.44.

Table 3.5 Amino acid content of the adult *Artemia*

Amino acid (mg/100gm)	CKF	VDA	TTJ	GMJ	TNM	TMM	SFB	Avg.
Ile	66.38 ± 1	69.39 ± 0.9	70.77 ± 0.8	65.4 ± 0.5	71.04 ± 0.76	64.52 ± 0.54	68.2 ± 0.78	67.96
Leu	95.33 ± 1.5	96.36 ± 1.5	102.63 ± 1.6	92.55 ± 1.3	101.18 ± 1.4	92.72 ± 1.23	92.38 ± 1.56	96.16
Tyr	34.12 ± 0.6	33.94 ± 0.6	35.74 ± 0.5	30.39 ± 0.23	34.03 ± 0.4	35.82 ± 0.32	32.94 ± 0.23	33.85
Met	18.58 ± 0.1	13.2 ± 0.5	19.86 ± 0.2	15.96 ± 0.12	18.36 ± 0.2	14.27 ± 0.2	22.97 ± 0.56	17.60
Phe	68.41 ± 0.8	70.25 ± 0.8	70.76 ± 0.9	63.18 ± 0.65	68.95 ± 0.5	69.81 ± 0.57	66.44 ± 0.87	68.26
Thr	71.23 ± 1	76.3 ± 1	76.45 ± 0.92	68.06 ± 0.88	70.59 ± 0.45	67.31 ± 0.88	73.04 ± 0.47	71.85
Val	72.07 ± 1.3	74.88 ± 0.4	76.55 ± 1	71.55 ± 0.92	77.44 ± 0.65	70.12 ± 0.48	73.01 ± 0.63	73.66
His	33.55 ± 0.5	37.89 ± 0.6	37.75 ± 0.56	36.45 ± 0.45	46.5 ± 0.8	31.27 ± 0.53	44.94 ± 0.63	38.34
Lys	107.7 ± 1.7	105.43 ± 1.8	122.18 ± 1.7	113.69 ± 2.2	114.15 ± 1.9	97.91 ± 1.54	116.35 ± 2.6	111.06
IAA	**567.37**	**577.64**	**612.69**	**557.23**	**602.24**	**543.75**	**590.27**	**578.74**
Asp	124.91 ± 1.6	128.65 ± 2	138.73 ± 2	98.02 ± 1.56	138.33 ± 1.9	110.64 ± 1.65	128.89 ± 2.9	124.02
Glu	192.48 ± 2.1	196.49 ± 2.6	208.36 ± 2.7	193.55 ± 2.45	201.32 ± 2.5	184.14 ± 2.45	186.91 ± 3.54	194.75
Ser	76.26 ± 1.1	82.37 ± 1.3	80.77 ± 1.2	76.43 ± 0.56	72.63 ± 0.55	70.18 ± 0.98	74.87 ± 0.64	76.22
Gly	71.53 ± 0.9	78.6 ± 1.1	72.56 ± 0.98	65.21 ± 0.87	74.8 ± 0.64	74.56 ± 0.45	61.92 ± 0.89	71.31
Arg	187.6 ± 2.6	197.9 ± 2.5	199.96 ± 2.76	184.62 ± 2.3	187.27 ± 1.23	183.66 ± 2.45	178.43 ± 2.65	188.49
Ala	71.98 ± 1	69.39 ± 0.8	76.9 ± 1.3	67.57 ± 0.87	77.96 ± 0.44	73.49 ± 0.87	69.18 ± 0.98	72.35
Pro	65.28 ± 0.6	66.46 ± 0.9	68.95 ± 0.8	59.19 ± 0.66	68.36 ± 0.9	70.47 ± 0.45	58.43 ± 0.77	65.31
Cys	0.95 ± 0.03	1.42 ± 0.03	4.04 ± 0.02	2.02 ± 0.06	1.41 ± 0.06	4.43 ± 0.05	5.39 ± 0.04	2.81
DAA	**790.99**	**821.28**	**850.27**	**746.61**	**822.08**	**771.57**	**764.02**	**795.26**
Total	**1358.36**	**1398.92**	**1462.96**	**1303.84**	**1424.32**	**1315.32**	**1354.29**	**1374.00**
IAA/DAA	0.42	0.41	0.42	0.43	0.42	0.41	0.44	0.42

All values are mean ± SD; SD; standard deviation.

Ile: Isoleucine, Leu: Leucine, Tyr: Tyrosine, Met: Methionine, Phe: Phenylalanine, Thr: Threonine, Val: Valine, His: Histidine, Lys: Lysine, IAA: Indispensable amino acid, Asp: Aspartic acid, Glu: Glutamine, Ser: Serine, Gly: Glycine, Arg: Arginine, Ala: Alanine, Pro: Proline, Cys: Cystine and DAA: Dispensable amino acid

3.4.1.5. Fatty acid composition

Saturated fatty acids (SFAs): Among the different developmental stages of the *Artemia*, total SFA in the adult stage was observed to be considerably higher than cyst and nauplii stages (Table 3.6). In the cysts, SFA was low in SFB and TNM strains (21.9 & 26.7%) while in others it ranged from 39.5 to 53.0 percent. In the case of nauplii, with the exception of SFB, TNM and TTJ strains, SFA content was much lower compared to cysts. Nauplii of GMJ and SFB strains exhibited considerably lower total SFAs (26.0 & 26.1%) than all the other strains, while total SFA in other nauplii are as follows - TNM (32.8%), CKF (38.7%), VDA (42.5%), TMM (45.3%) and TTJ (52.2%). Fatty acids 16:0 and 12:0 contributed 14-22% of the total SFA.

Monounsaturated fatty acids (MUFAs): Higher MUFA was observed in the nauplii and cyst (25 to 44%) stages compared to the adult (10 to 27%) stage. In all the stages of all the strains 18:1*n*-9 was found to be the prominent fatty acid compared to the rest. The cyst of TNM strain showed significantly higher MUFA (44.1%) compared to the reference SFB strain (34.5%) (Table 3.6). GMJ and VDA strains showed high MUFA content (45.4% & 40.8%) in the nauplii, as compared to the other indigenous and reference *Artemia* strains estimated (Table 3.6).

Table 3.6 Fatty acid composition (%TFA) of *Artemia* cyst from different locations

Fatty acids (%)	VDA	TTJ	GMJ	CKF	TMM	TNM	SFB
Saturated fatty acids							
12:00	15.12	23.36	24.64	0.64	1.39	0.19	0.25
14:00	3.06	ND	0.49	1.93	2.05	2.37	1.02
15:00	0.48	ND	0.21	ND	0.72	1.97	ND
16:00	23.93	10.74	19.21	19.71	27.38	16.79	17.32
17:00	ND	ND	1.61	2.46	1.24	1.19	0.89
18:00	8.13	6.61	4.52	4.44	4.18	3.53	ND
Others	2.30	7.67	0.00	.1656	2.42	0.50	2.46
∑ SFA	53.02	48.38	50.68	45.74	39.47	26.67	21.94
Monounsaturated fatty acids							
16:1n-7	11.01	21.07	7.56	16.07	10.44	13.86	6.02
18:1n-9	16.84	14.46	17.95	22.92	21.26	26.90	28.47
∑ MUFA	27.85	35.53	25.51	38.99	35.56	44.10	34.49
Polyunsaturated fatty acids							
18:2n-6	3.54	3.50	6.69	3.86	4.93	6.09	3.35
18:3n-6	1.53	0.50	0.07	3.05	2.18	0.41	4.20
18:3n-3	2.58	3.72	3.18	ND	3.06	2.82	11.07
20:2n-6	0.57	1.50	1.66	2.46	2.86	0.67	3.29
20:3n-6	2.01	1.40	ND	2.78	0.85	0.14	0.67
20:4n-6	4.21	1.20	2.00	ND	0.73	1.11	0.87
20:5n-3	1.63	2.30	7.61	1.18	1.28	7.05	6.12
22:5n-3	ND	0.05	0.23	0.18	0.09	0.04	0.15
22:6n-3	ND	1.65	1.02	0.54	0.84	0.07	0.19
∑ PUFA	16.07	15.82	22.48	14.05	17.14	18.53	29.91
Others	3.06	0.27	1.33	1.22	7.83	10.7	13.66
∑ n-3	4.21	5.37	14.04	1.72	5.27	9.98	17.53
∑ n-6	11.86	8.10	8.42	12.15	11.55	8.42	12.38
∑ C18PUFA	7.65	3.72	9.94	6.91	10.17	9.32	18.62
∑ C20PUFA	8.42	6.40	9.27	6.42	5.72	8.97	10.95
n-3/n-6	0.35	0.66	1.67	0.14	0.46	1.19	1.42
∑PUFA/∑SFA	0.30	0.33	0.44	0.31	0.43	0.69	1.36
EPA/AA	0.39	1.92	ND	ND	1.75	6.35	7.03
DHA/EPA	ND	0.72	0.40	0.46	0.66	0.01	0.03

ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; ΣPUFA: total polyunsaturated fatty acids; Data presented as mean values of three samples. ND = fatty acid identified as trace, but not integrated.

Polyunsaturated fatty acids (PUFAs): Considerable variation in the total PUFA content was observed between the different developmental stages as well as between the strains as described in the following sections.

Cysts: Total PUFA in the cyst ranged from 14.1 to 29.9 per cent (Table 3.6). The level of20: 5n3 in different strains ranged from 1.2 to 7.6%. Among the strains GMJ and TNM registered high levels (7.6 & 7.1% respectively) compared to the other strains. The 22:6n 3 was low in all the strains (0 to 1.7%) except GMJ which was 3.0%. Considerably low 20:4n 6 was observed in all the strains (0 to 1.2%) except in the VDA strain (4.2%). The 18:3*n*-3 formed the major fraction (11.1%) of the total PUFA in the SFB strain whereas its contribution was less (0 to 3.7%) in the native strains (Table 3.6). Essential PUFAs viz., 20:5*n*-3 and 22:6*n*-3 were high in GMJ strain (7.6 and 3.0%) when compared to the reference SFB strain (6.1 and 0.2%) and other indigenous strains studied.

Nauplii: Considerable variation in total PUFA was observed in the nauplii of the different *Artemia* strains evaluated (12.5 to 43.0%). Total PUFA was observed to be high in SFB (43.0%) and TNM (38.0%) strains mainly due to the large amounts of the fatty acid fractions of 18:3n 3 in SFB (26.3%) and 18:2n 6 in TNM (29.5%). The 20:5n 3 in the nauplii stage ranged from 1.3 to 7.3 per cent. It was high in the native strains such as TTJ and GMJ (5.1 & 7.3%) when compared to the reference SFB strain (1.9%). Except in TNM and TMM strains (1.71 & 1.58%), 22:6n 3 content was low in all strains including the reference SFB strain (0.18%). DHA/ EPA ratio was above 0.5 in VDA, TNM and TMM strains while it was low (<0.5) in the reference and other native strains studied (Table 3.7).

Table 3.7 Fatty acid composition (%TFA) of *Artemia* nauplii from different locations

Fatty acids (%)	VDA	TTJ	GMJ	CKF	TMM	TNM	SFB
Saturated fatty acids							
12:00	2.12	31.32	4.77	0.10	0.92	1.48	0.21
14:00	8.57	0.00	0.21	8.61	2.87	2.87	2.58
15:00	1.92	ND	0.16	1.68	2.76	0.93	ND
16:00	22.17	19.67	18.56	19.14	27.35	17.43	14.69
17:00	2.38	0.56	0.44	1.54	2.60	1.48	1.68
18:00	4.90	0.69	1.57	7.61	7.05	6.03	4.33
Others	0.00	0.00	0.00	0.00	1.44	2.22	0.59
∑ SFA	42.49	52.24	26.01	38.68	45.28	32.84	26.08
Monounsaturated fatty acids							
16:1n-7	16.71	4.88	10.20	8.99	8.21	1.02	5.26
18:1n-9	24.05	30.38	35.22	20.99	12.18	14.49	25.58
∑ MUFA	40.76	35.26	45.42	29.98	30.70	22.30	30.84
Polyunsaturated fatty acids							
18:2n-6	2.81	3.47	13.20	6.31	4.78	29.5	7.80
18:3n-6	1.92	0.47	1.57	0.07	2.58	1.27	0.50
18:3n-3	4.07	0.26	2.08	3.33	2,83	0.53	26.31
20:2n-6	0.76	1.50	ND	ND	0.29	0.06	5.17
20:3n-6	0.66	0.50	0.23	1.06	0.54	0.06	0.95
20:4n-6	0.26	0.77	1.38	0.93	1.53	1.33	ND
20:5n-3	1.29	5.10	7.25	3.22	1.59	1.30	1.88
22:5n-3	ND	ND	ND	ND	0.27	0.77	0.20
22:6n-3	0.69	0.64	ND	0.38	1.71	1.58	0.18
∑ PUFA	12.46	12.71	28.49	15.30	17.82	37.99	42.99
Others	4.29	-0.21	0.08	16.04	6.2	6.87	0.09
∑ n-3	6.05	6.00	9.33	6.93	6.40	4.18	28.57
∑ n-6	6.41	4.71	16.38	8.37	9.72	32.26	14.42
∑ C18PUFA	8.80	4.20	16.85	9.71	10.19	31.34	34.61
∑ C20PUFA	2.97	5.87	8.86	5.21	3.95	2.75	8.00
n-3/n-6	0.94	1.27	0.57	0.83	0.66	0.13	1.98
∑PUFA/∑SFA	0.29	0.24	1.10	0.40	0.39	1.16	1.65
EPA/AA	4.96	6.62	5.25	3.46	1.04	0.98	ND
DHA/EPA	0.53	0.13	ND	0.12	1.08	1.22	0.10

ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; ΣPUFA: total polyunsaturated fatty acids; Data presented as mean values of three samples. ND = fatty acid identified as trace, but not integrated.

Table 3.8. Fatty acid composition (%TFA) of *Artemia* adult from different locations

Fatty acids (%)	VDA	TTJ	GMJ	CKF	TMM	TNM	SFB
Saturated fatty acids							
12:00	29.03	3.10	5.49	13.20	18.92	0.75	7.16
14:00	4.71	40.05	28.67	0.95	0.88	5.00	9.81
15:00	0.88	1.81	1.76	ND	14.12	0.19	1.39
16:00	31.66	12.31	12.14	20.25	9.19	11.97	22.27
17:00	0.77	0.09	0.07	ND	0.61	4.86	ND
18:00	2.74	5.38	3.46	12.87	3.24	4.86	3.91
Others	0.00	0.00	0.00	3.8	0.95	1.12	3.71
∑ SFA	75.38	62.74	51.59	51.07	48.25	28.94	48.25
Monounsaturated fatty acids							
16:1n-7	0.99	3.71	5.29	1.15	10.07	5.24	5.10
18:1n-9	9.97	9.09	9.08	13.96	11.42	21.65	12.40
∑ MUFA	10.96	12.80	14.37	15.11	21.49	26.89	17.50
Polyunsaturated fatty acids							
18:2n-6	3.40	3.77	5.45	2.46	4.73	6.17	4.77
18:3n-6	ND	0.03	0.03	7.56	3.04	5.66	1.86
18:3n-3	1.97	1.40	2.13	2.73	4.93	2.34	3.71
20:2n-6	2.19	ND	ND	1.93	0.74	8.04	ND
20:3n-6	1.86	1.58	2.29	ND	0.61	0.28	1.29
20:4n-6	0.77	1.17	0.96	3.69	3.45	1.96	8.48
20:5n-3	0.88	6.26	7.92	6.95	1.42	3.55	6.89
22:5n-3	ND	0.09	0.27	1.32	0.14	0.14	ND
22:6n-3	0.22	0.18	1.06	2.99	1.96	0.14	1.48
∑ PUFA	11.29	14.48	20.11	29.63	21.83	28.65	28.48
Others	2.37	9.98	13.93	4.19	8.43	15.52	5.77
∑ n-3	3.07	7.93	11.38	13.99	8.45	6.17	12.08
∑ n-6	8.22	6.55	8.73	15.64	12.57	22.11	16.40
∑ C18PUFA	5.37	5.20	7.61	12.75	12.70	14.17	10.34
∑ C20PUFA	5.70	9.01	11.17	12.57	6.22	13.83	16.66
n-3/n-6	0.37	1.21	1.30	0.89	0.67	0.28	0.74
∑PUFA/∑SFA	0.15	0.23	0.39	0.58	0.45	0.99	0.59
EPA/AA	1.14	5.35	8.25	1.88	0.41	1.81	0.81
DHA/EPA	0.25	0.03	0.13	0.43	1.38	0.04	0.21

ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; ΣPUFA: total polyunsaturated fatty acids; Data presented as mean values of three samples. ND = fatty acid identified as trace, but not integrated.

Adults: Total PUFA in the *Artemia* adults ranged from 11.3 to 29.63 per cent, and it was high in CKF, TNM and SFB strains (29.6, 28.7, 28.5% respectively). The level of 20:5n-3 in GMJ and CKF strains were 7.9% and 7.0% respectively when compared to 6.9% of the SFB strain. Similarly, higher 22:6*n*-3 values were observed in CKF (3.0%) and TMM (2.0%) strains while in other strains it ranged from 0.2 to 1.5 per cent. However, 20:4*n*-6 content appeared to be high in SFB strain (8.5%) when compared to the native strains. Considerably higher EPA/AA ratio was recorded in GMJ (8.3) and TTJ (5.4) strains than the reference (SFB, 0.8) strain (Table. 3.8).

3.5. Discussion

Increasing demand for quality live feeds in the aquaculture industry stresses the need for identification, characterization and evaluation of novel strains of *Artemia* with better qualities. The present study revealed the significance of the indigenous strains of *Artemia* in terms of nutrient quality.

Hatching percentage and trehalose content of the cyst (5.8 to 11.5%) of the various strains showed a significant positive correlation (7.81) ($P<0.05$) (Fig. 3.2) indicating that the trehalose in the cysts acts as a stabilizing agent to protect the membrane proteins, thereby helping the cysts to survive the repeated dehydration-rehydration impacts and this would enhance the shelf life of the cysts (Clegg *et al.,* 2000; Wang *et al.,* 2010). This is evident from the case of the GMJ strain which had the highest trehalose content (11.5%) and an hatching percentage of 84.5%. The trehalose content appeared to diminish in the nauplii (4.1 to 7.6%) and the adult stages (3.3 to 4.8%) and is in agreement with the observations of Godelieve and Macrae, (2002).

An ideal starter diet for the larvae should have high level of soluble proteins to meet the requirement resulting from very high growth rate. Soluble proteins are easily broken down by intestinal proteases, increasing their availability for uptake by pinocytosis, and thereby enhancing the larval dietary protein uptake (Srivastava *et al.,* 2006). In the present study, both the nauplii and the adult *Artemia* were found to be equally high in terms of soluble protein. In the nauplii, it ranged from 27.3 to 48.1% compared to adult stages where it was from 28.8 to 48.1%. The cyst was found to havea low content of soluble protein (9.7 to 16.3%) (Fig. 3. 3) making it a less preferred option as a source of soluble protein. The high soluble protein content in the nauplii of the indigenous *Artemia* strains (TTJ and CKF) compared to the commercially available SFB strain points towards their nutrient superiority.

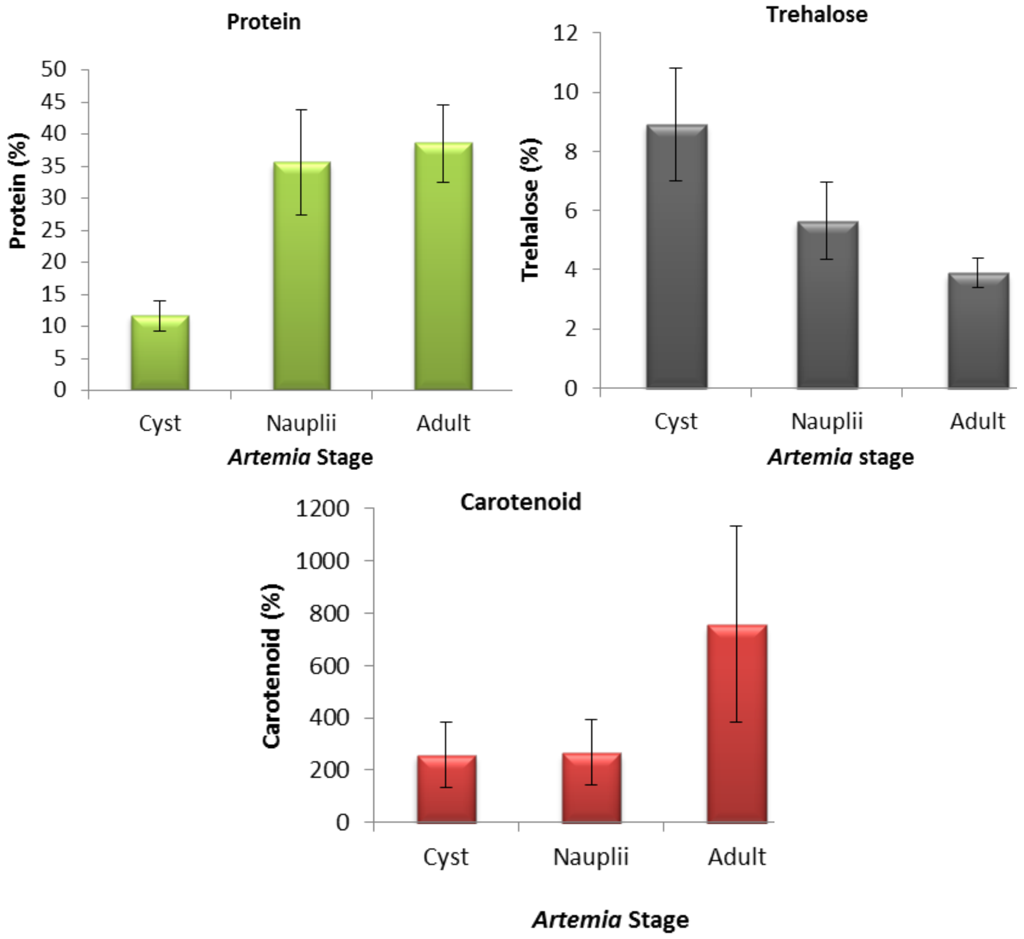


Fig.3.3 Mean values of Protein, Trehalose and Carotenoid content of cyst, nauplii and adult stages of indigenous strains of *Artemia*.

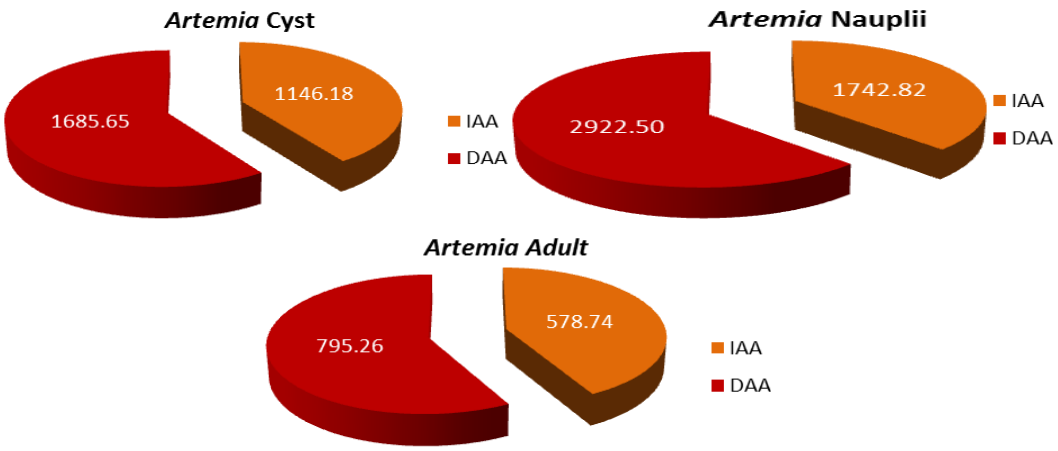


Fig. 3.4 Mean values of Indispensable Amino acid (IAA) and Dispensable Amino acid (DAA) content of *Artemia* cyst, nauplii and adult of indigenous *Artemia* strains.

Immediately after hatching, the larvae gets the essential amino acids from the yolk protein through protein hydrolysis pathway. When the yolk proteins are exhausted, the larvae have to obtain these essential amino acids through exogenous feeding. The amino acids in the proteins represent about 90% of total amino acids in *Artemia.* In the present study, high levels of IAAs were observed in the nauplii stage (1742.82 mg/100gm) when compared to the cyst (1146.2 mg/100gm) and adult stage (578.7 mg/100gm) of the *Artemia* (Fig. 3.4).

Among the various IAAsof the indigenous *Artemia*, the tyrosine, methionine and histidine were less when compared to the lysine and valine, irrespective of their origin and life cycle stage (Table 3. 3, 4 & 5). Among the three stages, cyst showed marginally high tyrosine content than the nauplii and the adult stages. In the case of DAAs, arginine, glutamine and aspartic acid contributed the major share in all the stages of *Artemia* while glycine, proline and cystine contributed the least. Limiting AAs such as threonine, leucine, arginine, lysine and histidine were observed in high levels in the nauplii stage than the cyst and adult stages of *Artemia* which has been reported by many workers (Conceição *et al.*, 1997, 2003; Aragão *et al.*, 2004). Similarly, methionine, another limiting AA was also found to be high in the nauplii stage (36.8 to 106.4 mg/100gm) when compared to the cyst and adult stages. Interestingly, among the strains studied, methionine showed significantly high values in TTJ, TNM, TMM and CKF strains (106.4, 94.5, 90.0 and 88.7mg/100gm) than the reference SFB strain (36.8mg/100gm) indicating the nutritional superiority of the native strains. Methionine forms the precursor of several compounds involved in important physiological functions in larvae (Conceição *et al.*, 1997). High levels of histidine, another IAA was observed in the nauplii (100.5mg/100gm when compared to the cyst and adult (57.0 and 36.3 mg/100gm) stages. Histidine level was also high in all the indigenous strains (81.3 to 145.0 mg/100gm) compared to the reference SFB strain (72.6mg/100gm), indicating their superior nutrient quality. Larval growth and feed conversion efficiency can be enhanced by feeding larvae with high essential dietary amino acids holding diets (Conceição *et al.*, 2003).

Independent of their origin, high carotenoid content was observed in the adult stage than the other stages of the *Artemia* strains studied. Variation in the carotenoid content in the developmental stages may be due to the metabolic rearrangements in the carotenoid pathways. For example, Browne *et al.*(1991) have reported that the high carotenoid content in adults may be due to the mobilization or accumulation of carotenoids especially trans-canthaxanthinsin to tissues especially gonads and the eggs. Feeding studies with β carotene by Hsu *et al.*, (1970) have revealed the ability of adult *Artemia* to bio-convert β carotene into echineone and canthaxanthin. Among the adults of various strains of *Artemia* studied, TNM and TMM strains showed high carotenoid level when compared to the reference strain (SFB). Since all the strains were reared in identical conditions, the presence of high carotenoids in the above strains reflects their genetic capability to synthesize more carotenoids from the food sources. These results also indicate the overall ability of *Artemia* to biosynthesize polar carotenoids from precursor molecules during the advancement of life cycle stages from cyst to adult. Hyperchromic shift inλ_{max} value of cyst and nauplii (from ε=9000,474 nm in the cyst to ε=12000, 77 nm in nauplii) also supports the presence of polar carotenoids like *β*-echineone and canthaxanthin in naupliiar stage.

The mean values for the total carotenoids in the cysts and the nauplii estimated across the strains were 259.5 µg/gm and 266.9 µg/gm respectively, and did not show any significant difference between the stages. The strain difference in the total carotenoid level of both cyst and nauplii were statically significant ($P<0.05$). It can be inferred that the indigenous strains viz, TNM and TMM with high carotenoid values, are promising alternatives to the imported strains in terms of nutritional values.

Pearson correlation analysis showed a positive correlation between the carotenoid and protein content in the different developmental stages of *Artemia* ($P<0.05$). This may be due to the conjugation of carotenoids with proteins to form carotenoprotein, and might involve the *in vivo* stabilization of proteins to protect carotenoids from photo-oxidation as suggested by Heras *et al.,* (2007). Velu *et al.,* (2003) observed that carotenoids have the ability to serve as the active prosthetic compounds in controlling the configuration and the stability of certain proteins. The carotenoprotein complexes present in the cuticle are also involved in the different photochemical processes, electron transport and enzymatic reactions in the crustaceans (Nègre-Sadargues *et al.*, 2000).

A considerable reduction in saturated fatty acid (SFA) was apparent in the nauplii when compared to the cyst stage in native strains. The SFA values increased during the development from nauplii to adult stage indicating the assimilation of SFA through feed (Fig. 3. 5). These SFA rich adult *Artemia* can be fed to the larvae in their later development stages to enhance the growth and metabolic activity because they are the storehouse of metabolic energy and excess SFA would be deposited as triglycerides in larvae which are easily catabolizable (Sargent *et al.*, 2002). In the present study, the fatty acid 16:0 forms the major share of the total SFA, as reported earlier (Moraiti-Ioannidou *et al.*, 2007).

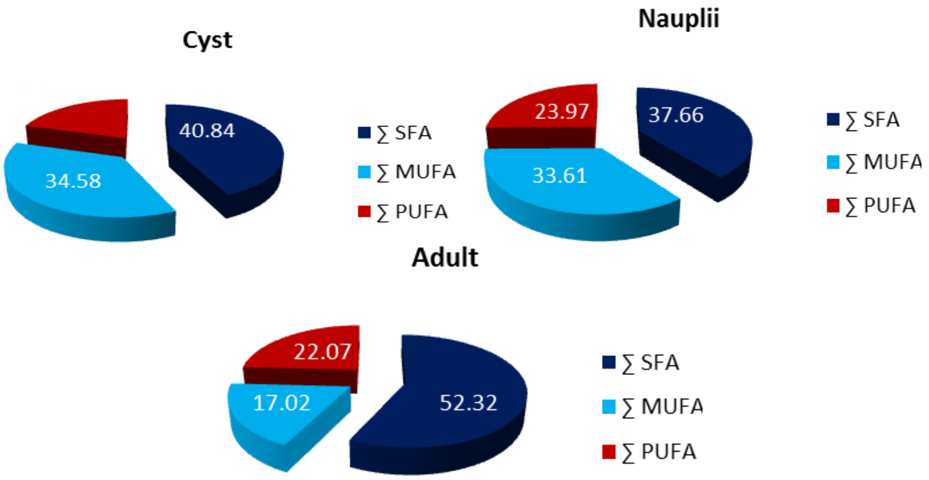


Fig.3.5 Mean values of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) of the *Artemia* cyst, Nauplii and Adult.

Monounsaturated fatty acids (MUFAs) in the nauplii of GMJ strain was significantly higher (45.4%) than the reference SFB strain (30.8%). It was significantly higher than the values reported for *Artemia persimilis* (33.1%) and other commercial *Artemia* strains (40.6%) (Villalta *et al.*, 2008; Liddy *et al.*, 2005). MUFA showed a substantial reduction in the adults (2.5 fold) when compared to the nauplii and cyst stages (Table 3.6) (Fig. 3. 6). Though 18:0 and 16:0 levels were high in adults, a corresponding increase in the levels of 18:1*n*-9 and 16:1*n*-7 level was not observed which may be due to the low titre of desaturase enzyme in adults. The fatty acid 16:1*n*-7 was observed in high proportions (11 to 21%) in the VDA, TTJ and CKF strains which may be due to the abundant occurrence of diatoms in the Indian hyper-saline habitats. A higher proportion of C_{18} MUFAs (*viz.*, 18:1*n*-9) and PUFAs (*viz.*, 18:2*n*-6, 18:3*n*-6, and 18:3*n*-3) in the *Artemia* cysts and nauplii are an indication of the nutrient rich feed consumed by their parents. This conclusion is supported by the fact that the microalgae present in the water column, are characterized by C_{18} PUFA (*viz.*, 18:2*n*-6 and 18:3*n*-3) (Volkman *et al.*, 1998).

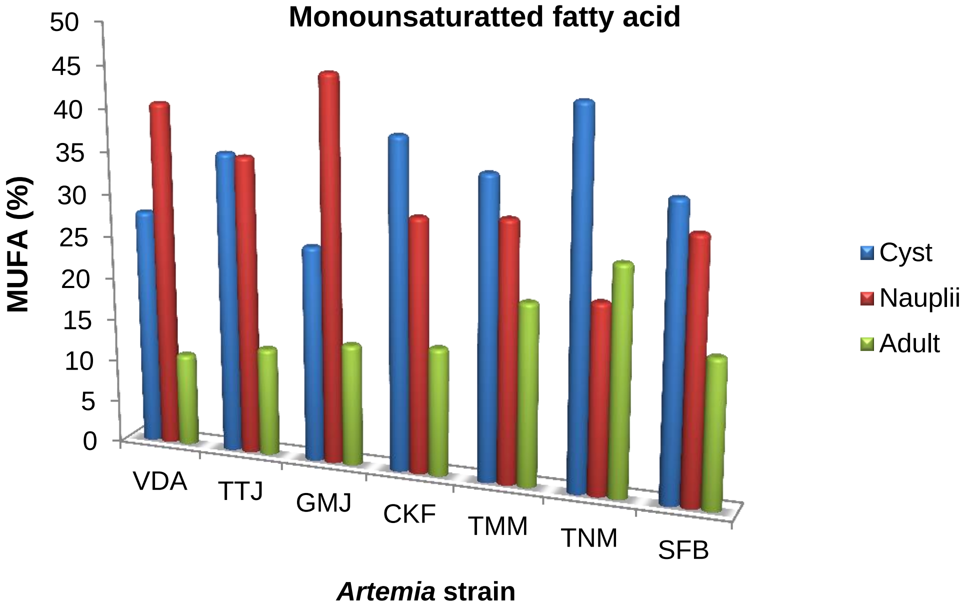
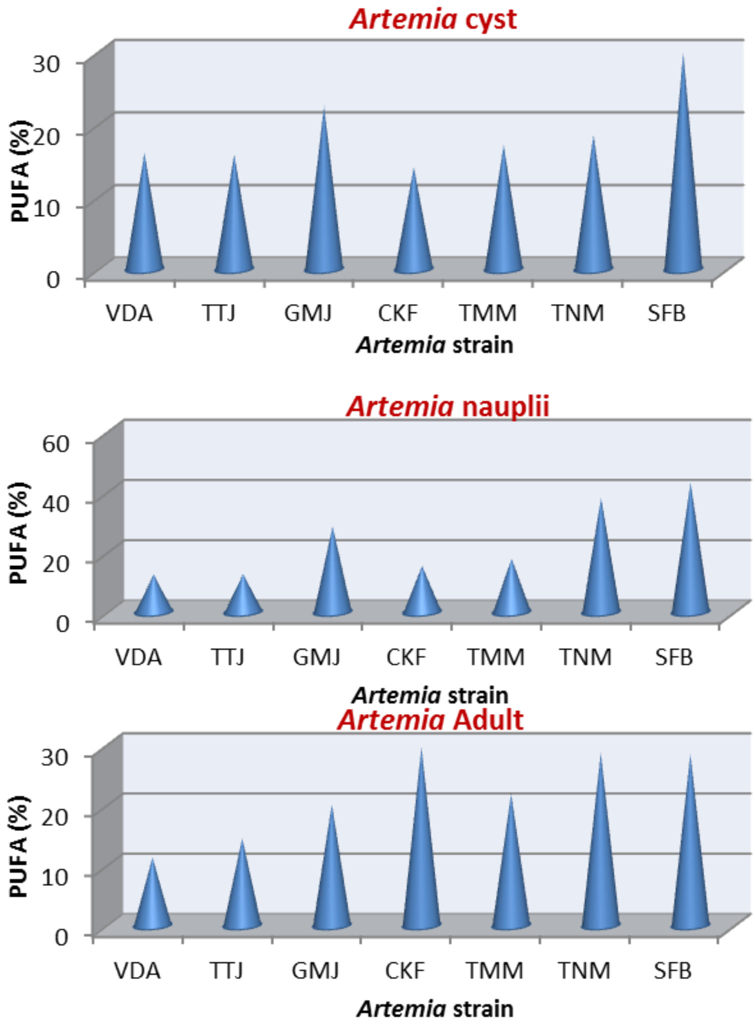


Fig. 3.6 Mean values of monounsaturated fatty acid (MUFA) content of indigenous and reference *Artemia* cyst, Nauplii and Adult.

In case of PUFA, only a few species have been reported to have the ability to bio-convert dietary fatty acids to PUFAs. The key issue in the fatty acid dynamics of *Artemia* is whether it can modify dietary fatty acids to essential PUFAs (Ruiz *et al.*, 2007). In nature, there is evidence of conversion of fatty acid 18:3*n*-3 to 20:5*n*-3 and then to 22:6*n*-3 by desaturase and elongase enzymes from the fatty acid precursors. The bioconversion of 18:2*n*-6 to 20:4*n*-6 was reported in insects, few freshwater fish species and *Artemia* sp. from the short chain fatty acid precursors under exceptional physiochemical conditions, but this phenomenon was reported to occur poorly in marine fish/ shrimps (Sargent *et al.*,2002).

Among the different stages of indigenous *Artemia* strains studied, high total PUFA was observed in the nauplii stage (24.0%) when compared to adult (22.1%) and cyst stages (19.1%). Nauplii of GMJ and TTJ (Fig. 3. 7) showed high 20:5*n*-3 content when compared to the reference and other strains evaluated. These values are higher than those reported for the enriched nauplii of *Artemia salina* (4.2%) and *A. franciscana* (2.5 to 5.1%) by Chakraborty *et al.*, (2007 and Immanuel *et al.*, (2007), indicating the superiority of the native strains with respect to essential PUFA.


Artemia cyst

Artemia nauplii

Artemia Adult

Fig. 3.7 Mean values of polyunsaturated fatty acid (PUFA) content of indigenous and reference *Artemia* cyst, Nauplii and Adult.

Marshall *et al.,* (2010) have opined that high content of essential PUFA is an important factor for determining the nutritional superiority. It can be inferred that GMJ and TNM strains have a higher rate of metabolic bioconversion of fatty acids from 18:3*n*-3 to 20:5*n*-3 indicating an innate biosynthesis ability/enhanced elongase-desaturase enzyme activity as suggested by Jaya-Ram *et al.,* (2011).

Though the reference SFB strain nauplii have a higher 18:3*n*-3 content (26.3%), the 20:5*n*-3 content in it was low (1.9%), indicating its poor ability for the bioconversion of 18:3*n*-3 into 20:5*n*-3. Bell *et al.,* (2003b) have identified the ideal ratio of 20:5*n*-3/20:4*n*-6 in *Artemia* nauplii as 5 or above for being used as live feed in larviculture. In the present study, the nauplii of VDA, TTJ, CKF and GMJ strains exhibited high 20:5*n*-3/20:4*n*-6 ratios (5.0, 6.6, 5.3, 3.5 and 6.6 respectively) indicating their superior nutritional quality.

Bell *et al.,* (2003a) have observed that 22:6*n*-3 is essential for the neural development and pigmentation in finfish larvae. But, studies by various investigators have shown that except in *Artemia tibetiana,* the 22:6*n*-3 is low in all other strains (Kara *et al.,* 2004). Among the strains evaluated in the present study, the 22:6*n*-3 was high in the nauplii of TMM, TNM, VDA and TTJ strains (0.64 to 1.71%) when compared to the reference SFB strain nauplii (0.2%). The trend was the same as reported for Greek *Artemia* nauplii (0.1 to 0.3%) and the enriched *Artemia salina* nauplii (0.1%) (Moraiti-Ioannidou *et al.,* 2007; Chakraborty *et al.,* 2007). Higher content of these essential PUFAs viz., 20:5*n*-3 and 22:6*n*-3 in the native *Artemia* strains revealed their nutritional superiority when compared to the commercially available strains.

The present study based on five nutritional indicators (soluble protein, carotenoid, trehalose, amino acid and fatty acids) in various indigenous strains of *Artemia* reveals their distinct nutritional superiority over the widely used commercial *Artemia franciscana* strain. Though different stages of *Artemia* (cyst, nauplii and adult) have been widely used as live feed, the nutritional superiority combined with the soft texture, swimming ability makes the nauplii stage, the most suitable one for feeding finfish and shellfish larvae. Among the various indigenous strains studied, the TMM strain (high IAA, DAA, TAA, carotenoid, trehalose, total PUFA and DHA) and the GMJ strain (high carotenoid, EPA and DHA) appeared to be the most promising candidate as live feed. Further studies on the possibility of using these *Artemia* as base strains for selective breeding programmes to develop better strains remains to be carried out.

3.6 Conclusions

The present investigation provides valuable insights into the nutritional variations in the cyst, nauplii, and adult stages of the *Artemia* strains from the hyper-saline habitats of India. The study revealed that the superior nutrient quality in terms of soluble protein, IAA, DAA, carotenoid and fatty acids in the native *Artemia* strains when compared to the widely used commercial strains. Such indigenous strains with higher nutritional profile can either be used directly as live feed through large scale farming, or may be explored as base strains for selective breeding programmes to develop better strains.

* * *

Chapter 4

Selective breeding for reduction of nauplii size in the indigenous *Artemia* strain

Vikas P.A, Sajeshkumar N.K, Thomas P.C, Kajal Chakraborty, Jayasankar J, Vijayan K.K

Selective breeding for nauplii size reduction in the naturalized *Artemia franciscana* from Indian Salinas. Communicated**.**

4.1. Abstract

The present study was aimed to develop an *Artemia* strain producing small nauplii through selective breeding. Fifteen generations of mass selection was carried out for the reduction of nauplii size in the indigenous *Artemia*, which was identified to be *Artemia franciscana* naturalized in the Indian Salinas. Mean values of life history traits, heritability of nauplii size, selection differentials, standardized selection differential, and predicted and realized selection responses were estimated from the full sib data. The selection response realized from fifteen generation of selection was 14.9 per cent reduction in nauplii length (from 517.0 ± 39.8 µm to 439.3 ± 27.0µm). Five per cent reduction in cyst size (from 224.83 ± 14.81 µm to 212.5 ± 9.5 µm) was realized as correlated response. Concurrently with the reduction in nauplii and cyst size, significant increase in cyst hatching percentage (10%) was also realized as correlated gain (from 54.4% to 64.58). Heritability estimates (h^2) were found to be generally very high. Heritability estimate for the first day nauplii length, pooled for the fifteen generations was 0.94 ± 0.27. The reduction

gained in nauplii length selection indicates the efficiency of selection. Since nauplii/cyst size and hatching efficiency along with the nutrient profile are the prime indicators that determine the suitability of *Artemia* as larval feed, the selectively bred *Artemia* of the present study make it a promising strain for larviculture activities.

Keywords: *Artemia; Cyst size, hatching percentage; heritability; selective breeding; small nauplii.*

4.2. Introduction

Aquaculture continues to grow more rapidly than all other animal food production sectors in the world. Production from aquaculture has outpaced population growth, with the annual average per capita supply from aquaculture increasing at the rate of 7.1 per cent (FAO 2010). Success of any aquaculture venture principally depends on the timely availability of suitable larvae and fingerlings of cultivable size in the required numbers and this can only be provided by a hatchery based larval production system. In hatcheries, live feeds play a key role in deciding the survival, growth and development of the larvae (Sorgeloos *et al.,* 2001). Live feeds can stimulate the feeding and improve the digestive process of the larvae than the artificial micro diets through its prey-predator interactions and exogenous digestive enzymes that aid in digestion (Kamarudin *et al.*, 1994; Baskerville-Bridges and Kling, 2000). Since larvae cannot biosynthesize the essential polyunsaturated fatty acids (PUFAs) namely 20:5n3 and 22:6n3 from its precursors, it has to be supplied through external diets especially live feeds (Sargent *et al.,* 2002).

Among the different live feeds, *Artemia* are widely used as the starter diet in larviculture, mostly because of its on-demand hatching ability, soft texture, motility and nutrient content. Although all stages of *Artemia* are suitable diet for diversified group of finfish and shellfish larvae, nauplii stage is considered as the most preferred larval diet. Hatching efficiency, nauplii size and nutrient profile are the foremost indicators that determine the suitability of the *Artemia* for larviculture. The nauplii size is the most important factor in larviculture. This is because most of the finfish larvae are small with narrow buccal openings, and therefore, exogenous foods of small size are needed one or two days after hatching by which time vitellinic reserves are depleted (de Barros and Valenti, 2003). Selection of the diet by the larvae depends to a great extent on the prey size rather than its nutritional quality. Pepin and Penney (1997) observed a positive correlation between the mouth widths of the larvae with the prey size. Live feed- larvae interactions have been studied in many fish species (Olsen *et al.,* 1999). According to Munk (1992), the prey size preferred by cod larvae is about 5% of its length. Fernandez-Diaz. *et al.,* (1994) reported that the preferred size of live and inert prey for gilthead sea bream larvae is a function of mouth size in the range of 0.1-0.8 times the mouth width. Marine larvae switch to bigger prey slower than their physical capacity to ingest larger prey (Pepin and Penney, 1997). In this scenario there is a need for reducing the size of the *Artemia* nauplii.

Heritability of the trait has an important role in the genetic gain realized from selective breeding since it determines the proportion of the selection differential that can be transferred to the progenies. Heritability is the proportion of the variance in a trait among individuals that is attributable to the differences in genotype. It in the narrow sense is the ratio of additive genetic variance to phenotypic variance. Heritability is a concept that summarizes how much of the variation in a trait is due to variation in genetic factors (Lush 1940). Frequently, heritability is used in reference to the resemblance between parents and their offspring. In this context, high heritability implies a strong resemblance between parents and offspring with regard to a specific trait, while low heritability implies a low level of resemblance (Wray, N. & Visscher, P. (2008). Lush, (1940) has used the intra-sire correlations or regressions of offspring on dam as a method of estimating heritability. Falconer (1960) defined heritability as the regression of breeding value on phenotypic value ($h^2 = b_{GP)},$ which is equivalent to the square of correlation between breeding values and phenotypic values ($h^2 = r^2_{GP}$).

Information on the selective breeding studies in *Artemia* is scanty. However, heritability estimates of hatching percentage, cyst size, growth rate, thermal stability and heat resistance have been reported, and these reports have indicated the superiority of genotypic influence over the phenotypic control on these traits (Tackaert *et al.*, 1987; Clegg *et al.*, 2000; Clegg and Trotman, 2002; Briski *et al.*, 2008). Half-sib analysis of the naupliar length data of *Artemia franciscana* by Shirdhankar and Thomas (2003) have shown heritability values of moderate magnitude.

Selective breeding in *Artemia franciscana* for reduction of the naupliar length by Shirdhankar and Thomas (2003) have shown that it is very much amenable to selection as the genetic gain realized was substantial. Clegg *et al,* (2000, 2002) reported that the changes in thermal stability and heat resistance may occur extremely rapidly using genetic selection in Vietnam strain of *Artemia*.

In this scenario, the objective of the present study was to develop a small nauplii producing *Artemia* by selective breeding. Generation's wise estimation of heritability of naupliar size, life history traits and hatching percentage were also made from the selectively bred *Artemia* and compared with the base generation.

4.3. Materials and methods

4.3.1. Collection of *Artemia* cysts

Artemia cysts collected from the hypersaline habitats of Kelambakam (CKF), Southeast region of the India ($12^0$47’ N - 80^0 13’ E) during 2008 were brought to the Central Marine Fisheries Research Institute, Cochin laboratory in hypersaline brine filled polyethylene bags. These cysts were cleaned and processed by biphasic flotation method and dried under sun.

4.3.2 Hatching and rearing

Base generation of *Artemia* (G_0) was developed by hatching the cysts (1gm Lr^{-1}) following the established procedure (Sorgeloos, 1986). Freshly hatched nauplii were harvested and stocked (5 individuals per mL) in cylindrical acrylic tanks containing 35 ppt seawater (10Lr). Optimum temperature of 25 ± 1^0C, with mild aeration and photoperiod of 12h D: 12h L were maintained in all the three rearing tanks. Microalgae, *Isochrysis galbana* (30 X 10^4 cells ml^{-1}) taken from the microalgae culture facility of Central Marine Fisheries Research Institute, India were used as the feed (two times a day) for rearing *Artemia* daily. *Artemia* tanks were monitored daily and ten percent of the culture medium was exchanged daily using fresh seawater.

4.3.3 Selective Breeding

Mass selection was carried out following the established method described by Falconer, 1981. Freshly hatched *Artemia* nauplii (about 1.5 lakhs) were used as base generation, and the small individuals (15,000 constituting about 10%) were selected using 500 mesh filtering unit. Selected individuals were restocked (1 individual per mL) in to round acrylic tanks (12Lr) containing seawater (35 ± 2‰) for further rearing. The selected and unselected individuals were reared separately under standard culture conditions described above.

By the age of fourteen days, males and females of both the selected and unselected populations started to form riding pairs. From the selected population, the precopulatory pairs (1,000 pairs) were selectively harvested and stocked (1 precopulatory pairs per 20 mL) in cylindrical acrylic tanks (20L) and reared under uniform conditions.

Riding pairs started to produce first generation progenies (nauplii) from the third day (D3) of stocking. These progenies were collected and restocked in 3Lr plastic containers (2individuals mL^{-1}). Small progenies were selected to produce the next generation (G_2). Likewise, progeny collection and selection process was continued up to eighth day (D8). The selected *Artemia* were finally pooled and reared in 50Lr circular acrylic tanks (5 individual per mL). From the offspring produced by G_2, small nauplii were further selected as described earlier to produce the next generation (G_3). Selection for small size nauplii were carried out for fifteen generations (G_1 to G_{15}) using different filtering unit (480, 450 and 400µm).

4.3.4. Recording and analysis of nauplii length, cyst biometry and hatching percentage

Nauplii length: Nauplii length or first day length (FDL) of the generations (G0 to G15) of the selected animals were recorded from the representative samples using a microscope attached with DIGI EYE 330 camera and software (Dewinter Bio-wizard, India) and the mean values were estimated generation wise.

Cyst biometry: The selectively bred *Artemia* strain (SBAS) biomass was scaled up in one ton fiber-reinforced plastic tanks (FRP) following the standard culture conditions. Thereafter the salinity of the culture medium was increased slowly (10 ‰ daily $^{-1}$) to 250 ‰ to induce the oviparous reproduction in the SBAS. The newly formed cysts were collected, cleaned, processed, and dried under the shade and stored (4^0C) until further analysis. To estimate the cyst biometry, cysts were incubated in fresh water at 28^0C for 2 h for hydration and fixed with Lugol's Iodine solution to arrest further development and retain its circular shape. The diameter (µm) of the 500 representative cyst samples from each location was measured using a microscope attached with DIGI EYE 330 camera with the Dewinter software (Biowizard). Cyst biometry was compared with the reference strains such as *A. franciscana* (SFB), *Artemia salina* (ASL) *Artemia tibetiana* (TBS), *Artemia* Vietnam strain (VVC) and also with the native *Artemia* strains (CKF, GMJ, TTJ, TMM, VDA, and TNM).

Hatching percentage: Hatching percentage of the SBAS were analyzed and compared with the reference *Artemia* and other indigenous *Artemia* collected from different hypersaline habitats of India viz., Vedaranyam (VDA), Tuticorin (TTJ), Marakanam (TMM), Tamaraikulam (TNM) and Gujarat (GMJ). *Artemia* cysts were individually incubated in micro plates (96 well) having seawater (35‰) at room temperature, and kept under light. The plates were observed hourly after 8 hours of incubation for nauplii. Hatching percentage (H %) of the cysts was calculated from the data as follows: hatching % = (number of nauplii hatched /total number of cysts) X 100.

4.3.5. Heritability estimation

Heritability of the selected trait was estimated for each generation. Heritability estimate from the data generated through pair mating. Generation wise heritability estimates were then pooled and mean heritability was estimated.

The variance component analysis was used to estimate sire component of variance, and heritability was estimated from it. The linear statistical model used was

Y_{ik} = $\mu + P + e_{ik}$

Where,

Y_{ik} = Observation of the k^{th} progeny of the i^{th} sire

μ = Overall mean

P_i = Effect of i^{th} sire, where i= 1, 2, 3…..P

e_{ik} = Random error attributed to individuals, assumed to be normally and independently distributed with mean zero and variance $\sigma^2 e$.

The degree of freedom (D.F.), sum of squares (SS), mean sum of squares (MS) and expected sum of squares (EMS) used for estimation of heritability are given below:

Analysis of variance

Source of variation	D.F.	SS	MS	EMS
Between pairs	P-1	SS_P	MS_P	$\sigma^2_W + K_1 \sigma^2 P$
Between progeny within pairs	n-P	SS_W	MS_W	σ^2_W

Where,

P = Total number of pairs

N = Total number of progeny

K_1 = Average number of progeny per sire

σ^2 = Pair component of variance

σ^2_W = Error variance component

Computational Formula

Sources of variation	Sum of squares	Mean squares
Correction terms (C.T.)	$\frac{Y\ldots^2}{n_i}$	--------
Between pairs	$\sum \frac{Y\ldots^2}{n_i}$ –C.T.	$MS_P = SS_P / S\text{-}1$
Progeny within pair	$\sum_i \sum_j Y_{ij}^2 - \sum \frac{Yi}{n_i}$	$MS_W = SS_W / n. – P$

Estimation of variance and heritability

$$\sigma^2_W = MS_W$$

$$\sigma^2_P = \frac{MS_P - MS_W}{K1}$$

$$h^2_P = \frac{2\sigma^2_P}{\sigma^2_P + \sigma^2_W}$$

The value of K_1 was calculated from the following formula:

$$K_1 = \frac{1}{P-1} \left[n. - \frac{n_i^2}{n.} \right]$$

Standard error of heritability was calculated as per Swinger *et al*., (1964) using the following formula.

$$S.E.\ h^2 = 2\sqrt{\frac{2(n-1)\,(1-t)^2\,[1+K_1-1)\,t\,]^2}{K_1^2\,(n.P)\,(P-1)}}$$

Where,

't' is interclass correlation

$$t = \frac{\sigma^2_P}{\sigma^2_P + \sigma^2_w}$$

The heritability estimates were pooled over generations, following the procedure of Enfield *et al.*, (1966). The formula for pooling the estimate is as follows:

$$\text{Pooled } h^2 = \frac{\dfrac{h_0^2}{V_0} + \dfrac{h_1^2}{V_1} + \dfrac{h_2^2}{V_2} + \dfrac{h_3^2}{V_3} + \ldots\ldots \dfrac{h_n^2}{V_n}}{\dfrac{1}{V_0} + \dfrac{1}{V_1} + \dfrac{1}{V_2} + \dfrac{1}{V_3} + \ldots\ldots \dfrac{1}{V_n}}$$

$$\text{S.E. of Pooled } h^2 = \frac{1}{\dfrac{1}{V_0} + \dfrac{1}{V_0} + \dfrac{1}{V_0} + \dfrac{1}{V_0} + \ldots\ldots \dfrac{1}{V_0}}$$

Where,

h_0^2, h_1^2, h_2^2, h_3^2, h_4^2, ……… h_n^2 are the heritability of character in the corresponding generation G_0, G_1, G_2, G_3, G_4, ……… $G_{n.}$

$V_{0,}$ $V_{1,}$ $V_{2,}$ $V_{3,}$ $V_{4,}$ V_n are the squares of standard error of corresponding heritability's.

4.3.6. Selection differentials

Selection differentials were calculated as the difference between the mean of the selected individuals who has parented the next generation and the mean of the population before selection of the parents (Falconer, 1960). Standardized selection differential was estimated following the method described by Falconer, 1981.

$$\text{Standardized selection differential (i)} = \frac{\text{Selection differential}}{\text{Phenotypic standard deviation}}$$

Predicted response and selection gain in each generation was estimated from the full sib data following the method described by Falconer, (1981).

Predicted genetic response (R) = i σ^2p h^2

R = Average predicted response per generation

i = Standardized selection differential

σ^2p = Phenotypic standard deviation of the trait under selection

h^2 = Pooled heritability of selected trait

Pooled heritability was used for prediction of response since it is supposed to be more accurate than individual generation estimates (Kinney and Shoffner, 1967).

4.3.7. Statistical analysis

Generation-wise means of all parameters were examined for significance by analysis of variance (ANOVA) using the Duncan multiple tests by SPSS programme 13.0 (SPSS Inc, Chicago, USA).

4.4. Results

4.4.1. Phenotypic parameters

Morphological observations revealed the length of the freshly hatched *Artemia* nauplii within the base generation (G0) ranged from 400.0 µm to 570.0 µm with a mean value of 517.0 ± 39.8 µm (Table 4.1.).

Table 4.1 Generation wise mean nauplii length (µm) in selectively bred *Artemia*

Generation	Mean nauplii length with SD* (µm)
G0	517.0 ± 39.8^{a}
G1	514.6 ± 20.5^{ab}
G2	504.7 ± 38.5^{abc}
G3	501.7 ± 20.3^{c}
G4	491.0 ± 38.7^{cd}
G5	490.1 ± 19.4^{cd}
G6	482.5 ± 23.1^{de}
G7	477.1 ± 27.1^{ef}
G8	471.4 ± 27.1^{efg}
G9	464.1 ± 30.1^{fgh}
G10	463.4 ± 24.8^{fgh}
G11	459.4 ± 21.4^{gh}
G12	454.5 ± 29.1^{hi}
G13	452.2 ± 25.0^{hijk}
G14	444.4 ± 31.8^{ik}
G15	439.3 ± 27.0^{k}

* SD= Standard Deviation
Values with same superscript are not significantly different at ($P>0.01$).

4.4.2. Heritability estimates of nauplii length

Heritability estimate of the *Artemia* nauplii (first day length) are presented in Table 4.2. Heritability estimates of the selected *Artemia* showed generation to generation variations. Heritability was 0.99 ± 0.36 in the base generation while it varied between 0.36 and 1.64 in other generations. Though, the heritability estimates and the standard errors associated with individual generations varied widely the pooled heritability and standard error of the selected trait was 0.96 ± 0.01 (Table 4.2).

Table 4.2 Heritability estimate and standard error of the *Artemia* nauplii (first day length)

Generation	Heritability	Standard error
G0	0.99	0.36
G1	1.10	0.30
G2	0.73	0.39
G3	1.27	0.24
G4	0.47	0.18
G5	0.98	0.35
G6	1.09	0.28
G7	0.31	0.23
G8	0.36	0.31
G9	1.36	0.24
G10	1.21	0.29
G11	1.15	0.22
G12	1.64	0.21
G13	0.42	0.28
G14	0.53	0.30
G15	1.46	0.19

4.4.2.1. Selection differentials

Mass selection was practiced in the *Artemia* nauplii to bring about change in nauplii size. Selection differentials and standard selection differential are presented in Table 4.3. Selection differential in the base generation was -25.97 and it showed variations over the generations. Selection differentials ranged from -33.17 to -8.66 µm in other generations. Lowest selection differential was noticed in G14. Standard selection differential was -0.65 at the base generation and it showed generation wise variations and ranging from -0.95 to -0.27. Phenotypic standard deviation of the different generations is illustrated in Table 4.3. Base generation showed highest phenotypic standard deviation (39.84 µm) followed by G4 (38.75 µm) and G2 (38.59 µm).

Table 4.3 Selection differential of length (μm), Phenotypic standard deviation (μm) and Standardized Selection differential of the fifteen generations of selected *Artemia*

Generation	Selection differential (μm)	Phenotypic standard deviation (μm)	Standardized Selection differential
G0	-25.97	39.84	-0.65
G1	-33.17	35.52	-0.93
G2	-29.98	38.59	-0.78
G3	-32.54	34.38	-0.95
G4	-24.17	38.75	-0.62
G5	-25.78	27.48	-0.94
G6	-21.26	23.15	-0.92
G7	-16.52	27.19	-0.61
G8	-13.73	27.10	-0.51
G9	-10.19	30.13	-0.34
G10	-11.43	24.86	-0.46
G11	-11.82	21.44	-0.55
G12	-14.19	29.19	-0.49
G13	-14.02	25.09	-0.56
G14	-8.66	31.84	-0.27
G15	NA	27.09	NA

4.4.2.2. Response to selection

The generation wise mean values of nauplii length with standard deviation are presented in Table 4.1. The mean nauplii length was 439.3±27.0 µm, after fifteen generations of selection, as against 517.0±39.8 µm in the base generation. A gradual decrease in nauplii length was noticed during the selection process. Mean nauplii length was 514.6±20.5 µm in G1 while it was 504.7±38.5 µm in G2, likewise reduced in other generations also. Selection gain was -2.40 µm in G1 while it reduced sharply in G2 to -9.86 µm maximum selection gain was noticed in G4 (-10.70 µm) and minimum was in G10 (-0.76 µm). Selection gain in the fifteenth generation (G15) was -5.13 µm. Cumulative selection gain after the fifteen generations of selection was 77.67 µm Table 4.4.

Table 4.4 Predicted, realized and cumulative gain (µm) in nauplii length of *Artemia* from fifteen generations of selective breeding

Generation	Predicted gain (µm)	Realized gain (µm)	Cumulative gain (µm)
G0	-24.93	NA	NA
G1	-31.84	-2.40	-2.40
G2	-28.78	-9.86	-12.26
G3	-31.24	-2.97	-15.23
G4	-23.20	-10.70	-25.93
G5	-24.75	-0.95	-26.89
G6	-20.41	-7.61	-34.49
G7	-15.85	-5.33	-39.82
G8	-13.18	-5.78	-45.60
G9	-9.79	-7.21	-52.81
G10	-10.97	-0.76	-53.57
G11	-11.35	-3.94	-57.51
G12	-13.63	-4.96	-62.47
G13	-13.46	-2.31	-64.78
G14	-8.31	-7.76	-72.54
G15	NA	-5.13	-77.67

4.4.3. Correlated response

Cyst size: A reduction in cyst size was realized in the selectively bred *Artemia* strain (SBAS) as a correlated response. After 15 generations of selection the cyst size in the selected line got reduced to 212.5 ± 9.4 µm from 224.83 ± 14.81 µm in the base generation. It was also smaller than reference strains VVC (218.26 ± 10.84 µm), SFB (222.0 ± 14.5 µm), ASL (261.7 ± 17.4 µm) and TBS (319.4 ± 24.7 µm) as well as the other indigenous strains studied (236.4 to 219.6 µm) (Table 4.5.). Duncan's multiple range test categorized *Artemia* strains based on the cyst size into eight different homogeneous subsets. SBAS formed the first sole group with lowest cyst size, while the reference VVC and SFB strains and the native TMM strains formed the second group with marginally higher cyst size (218 to 221 µm). Other native strains viz, VDA and

TNM, and exotic species like ASL and TBS which possessed significantly higher cyst sizes (231.56, 236.38, 261.72 and 319.39 respectively) formed different subsets (Table 4.5.).

Hatching percentage: Selective breeding for nauplii size reduction resulted in a correlated increase in the hatching percentage also. At the end of 15 generations of selection the hatching percentage in the selectively bred strain was 64.58% as against 54.4% in the base generation which is collected from wild (Fig. 4. 1). It was higher than other indigenous strains except GMJ (84.52%) and exotic strains except SFB (72.22%).

Table 4.5 Mean cyst diameter of the base, selectively bred *Artemia* strains (SBAS) and the different reference *Artemia* strains

***Artemia* strain**	**Mean with SD[1] (μm)**	**SE [2]**
Base generation[3]	224.83 ± 14.81^{a}	1.30
SBAS[4]	212.49 ± 9.418^{d}	1.41
TTJ[5]	223.35 ± 11.57^{ca}	0.85
TMM[6]	219.63 ± 10.99^{bc}	1.54
VDA[7]	231.56 ± 15.61^{e}	1.17
TNM[9]	236.37 ± 19.00^{f}	2.03
GMJ[10]	226.16 ± 14.01^{a}	1.42
VVC[8]	218.26 ± 10.84^{b}	1.16
ASL[11]	261.72 ± 17.35^{g}	1.66
TBS[12]	319.39 ± 24.74^{h}	2.41
SFB[13]	221.95 ± 14.45^{bca}	1.46

[1] SD: Standard Deviation, [2] SE: Standard Error, [3]CKF/ Base strain: Kelambakam, [4] SBAS: Selectively Bred *Artemia* Strain, [5] TTJ: Tuticorin, [6] TMM: Marakanam, [7] VDA: Vedaranyam, [8] VVC: *Artemia* Vietnam strain, [9] TNM: Tamaraikulam , [10] GMJ: Mithapur- Gujrat, [11] ASL: *Artemia salina,* [12] TBS: *Artemia tibetiana* and [13] SFB: *A. franciscana.*
Values with same superscript are not significantly different at (P>0.01).

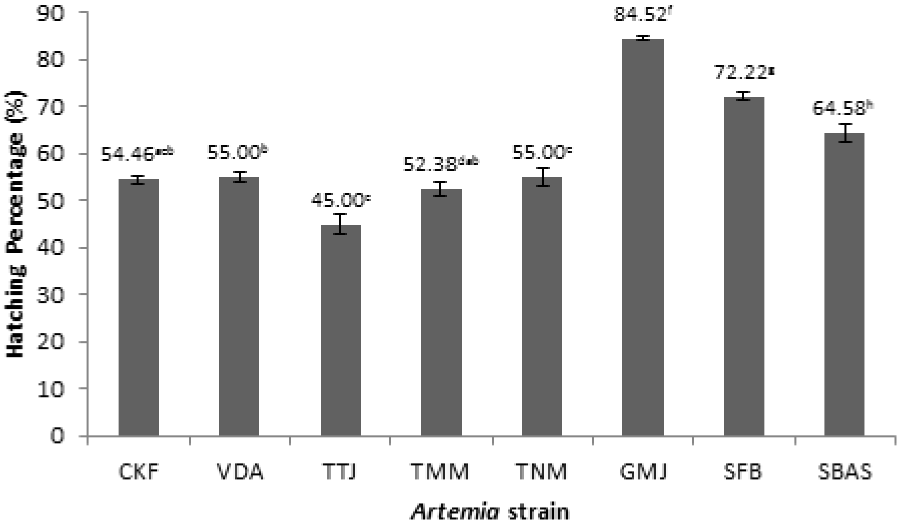


Fig 4.1 Hatching percentage of different *Artemia* strains

CKF/ Base strain: Kelambakam, VDA: Vedaranyam, TTJ: Tuticorin, TMM: Marakanam, TNM: Tamaraikulam , GMJ: Mithapur- Gujrat, SFB: *A. franciscana* and SBAS: Selectively Bred *Artemia* Strain.
Column wise vales with same letter (a, b, c, d, e, f, g, h) superscript are not significantly different ie, ($P>0.01$).

4.5. Discussion

The genetic studies in *Artemia* are limited to cytogenetics, genetic diversity, molecular taxonomy, phylogenetic analysis, etc. There is an apparent knowledge gap in quantitative genetics of the *Artemia* since only few attempts to study quantitative genetic parameters and quantitative genetic manipulations have been made hitherto.

In the present study, 14.9 per cent reduction in the nauplii length was realized from fifteen generations of selective breeding for size reduction in the indigenous *Artemia.* Concurrently, 5 per cent reduction in the cyst size and 10% increase in hatching percentage were also realized as correlated response in the selectively bred *Artemia* strain.

The nauplii length in the SBAS could be brought down to 439.3±27.0μm from 517.0±39.8 μm in the base generation through fifteen generation of mass selection. Size-wise, it is smaller than the nauplii of the commercial strain of *Artemia franciscana* (502.6±97.13) hatched along with the selected strain, and naupliiar size reported for *A. franciscana* (487.07 μm and 490.67 μm) by Shirdhankar *et al.*, (2004). Needless to say it is smaller than the naupliiar size reported for various *Artemia* species globally viz., *Artemia franciscana*, Greek *Artemia* sp. (507.4 to 455.0 μm) (Moraiti-Ioannido *et al.*, 2007), *Artemia urmiana* (466.3 to 505.0 μm) (Abatzopoulos *et al.*, 2006), Spanish Lamata *Artemia* (469.2 μm) (Hontoria, 1990), Italian Margherita di Savoia *Artemia* (517.0 μm) (Vanhaecke and Sorgeloos, 1980), Portugal Samouco *Artemia* (503.5 μm) (Vilela *et al.*, 1987) and *Artemia tibetiana* strain (667.0 μm) (Abatzopoulos *et al.*, 1998). The Italian Tore Colimena strain (422.7 μm) has the smallest nauplii length (Stappen, 2005).

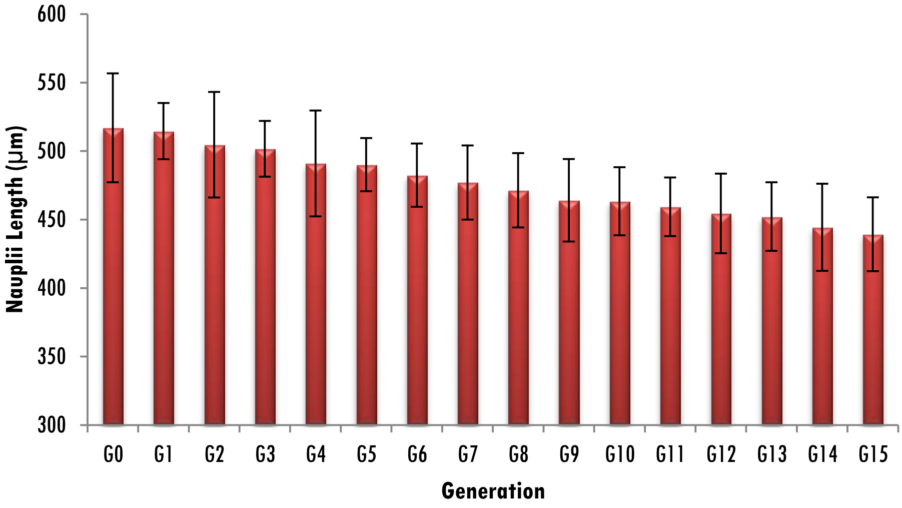


Fig 4.2 Generation wise nauplii length of *Artemia* under selection

G0: Base generation, G1: Generation1, G2: Generation2, … , G15: Generation15Mean values with standard deviation is represented in the graph

Correlated response by way of reduction in the cyst size and increase in hatching percentage were also realized in the selectively bred *Artemia* strain. Selection has resulted in 5% (-12.34µm) reduction in cyst size than that of base generation. SBAS cyst was found to be smaller than that of various *Artemia* strains reported worldwide viz., *A. urmiana* (262.7 to 286.6 µm) (Abatzopoulos *et al.,* 2006), Srilankan *Artemia* sp. (248.7 to 267.9 µm) (Kuruppu *et al.,* 1995), Algerian *Artemia* sp. (236.0 µm) (Kara *et al.,* 2004) and *A. parthanogenetica* (260.0, 244.9 µm) (Hontoria, 1990; John *et al.,* 2004). The reduction gained in cyst size and nauplii length after fifteen generations of selection indicates the efficiency of selection. Phenotypic differences observed among the offspring were due to genetic differences among parents pursuant to selection because all were cultured under the same environmental conditions. Size reduction following the selective breeding is brought about through changes in gene frequencies at loci that influence the selected character (Falconer, 1981). Selection induces a change in gene frequency by separating the individuals into large and small groups with a difference in gene frequencies (Liang *et al.,* 2010), from which the small groups were selected for developing the SBAS.

4.5.1. Heritability of the selected trait

Trait under selection was nauplii size and the full-sib values of nauplii length were used to estimate the heritability. Higher heritability (h^2) estimates were obvious in the different generations of *Artemia* subjected to selection in the present study. Heritability estimated from the full sibs produced from pair mating is the heritability in the broad sense and it represents the ratio of the total genetic variance to the total phenotypic variance. The total genetic variance includes additive genetic variance, variance due to dominance deviation and epistatic interaction (Shirdhankar, 1999), and hence are of higher magnitude than the estimate from half-sib data. Lester, (1988) have reported that the maternal effects and genetic difference among families may also result in an inflated value for heritability estimated from full sib data resulting from pair mating.

Though the pooled h^2 estimates for FDL in the SBAS was high (0.96 ± 0.01), it was lower than the h^2 estimates of *A. franciscana* strain (1.33 ± 0.04, 1.4 ± .04 and 1.3 ± .04) as reported by Shirdhankar and Thomas (2003). High heritability estimates values observed in full sibs from sire dam pair mating may be due to a substantial quantum of dominance deviation and / or epistatic interaction and maternal effects in the population under study as suggested by Shirdhankar, (1999) and Lester, (1988). Briski *et al.*, (2008) observed wide variations in h^2 values in the *Artemia* sp. subjected to selection (0.11 to 0.95). Reports of h^2 estimate beyond the normal theoretical limits in several species are not uncommon in the literature (McLaren *et al.*, 1978; Durborow *et al.*, 1985). Lester, (1988) suggested that the maternal effects and genetic differences among the families can result in inflated h^2 estimates from full sib data as evidenced in the early growth phase of *Penaeus stylirostris* (1.31 ± 0.62 to 0.64 ± 0.58). Falconer, (1955) has observed that variations in the h^2 often occur in generations, but they do not follow any particular trend as reported earlier. According to Wickins (1981), ectotherms aquatic animals generally lack sophisticated endogenous homeostasis mechanisms like mammals and birds possess, and because of this environment holds profound effect on phenotypic expression of the individual’s genotype.

The h^2 estimates of the FDL from the present work clearly indicate that the genetic effect on the nauplii length of *Artemia* sp profound. A substantial portion of the variance in the population is due to additive genetic variance, which is reflected in the cumulative selection gain of the mass selection programme. However, the higher h^2 values indicate that non-additive genetic factors are also contributing substantially to the total genetic variance.

Hatching percentage is an important quality indicator which decides the potential of the *Artemia* strain for aquaculture application. The present study shows that the cyst hatching percentage has significantly improved after the selection (10% increase) when compared to the base generation. The SBAS had higher hatching percentage (64%) than the unselected indigenous strains studied (45 to 55%), except the GMJ strain and exotic SFB strain of *A. franciscana.*

Nauplii/cyst size, hatching efficiency and nutrient profile are considered as the prime indicators that determine the quality of the *Artemia* strains for larviculture. The selectively bred *Artemia* strain developed from the present study fulfills all the above requirements making it a promising candidate strain for larviculture activities.

4.6. Conclusion

Present study reports the reduction in nauplii length by 14.9% as the direct response to fifteen generations of mass selection for nauplii size reduction in indigenous *Artemia*. Reduction of cyst size (5%) and increase in the hatching percentage (10 %) were also realized as correlated response. The selectively bred *Artemia* strain developed from the present work has small sized nauplii and cyst size and high hatching efficiency making it a promising candidate strain for larviculture. Further studies are required to validate the performance of the strain in field conditions.

* * *

Chapter 5

Biochemical and biometrical evaluation of *Artemia nauplii* enriched with Microalgae

P. A. Vikas, Kajal Chakraborty, N. K. Sajeshkumar, P. C. Thomas, N. K. Sanil and K. K. Vijayan (2012).
Unraveling the effects of live microalgal enrichment on *Artemia* nauplii. ***Indian Journal of Fisheries***, 59(4): 111-121, 2012.

5.1. Abstract

Artemia nauplii, in spite of its deficiency in some of the essential nutrients, are used extensively in larviculture. Enrichment of *Artemia* nauplii using various nutrients is generally practiced to enhance their nutrient profile to the required level. The present study examines the effect of enrichment of*Artemia* nauplii with live microalgae *Pavlova viridis, Isochrysis galbana, Nannochloropsis oculata, Dicrateria inornata* and a commercial enrichment diet, Algamac 2000on its fatty acid profile, growth, the survival rate and salinity tolerance. Fatty acid profile of the enriched nauplii was evaluated at different time intervals. The study revealed that five to seven hour of enrichment with live microalgae diets can significantly improve the essential PUFA content while keeping the nauplii size at minimum. Essential fatty acids *viz.,* 20:5n3 and 22:6n3 in the microalgae enriched nauplii showed an incremental trend, and reached the maximum between the 5th and 7th hour of enrichment. Significant variations observed in the fatty acid profile of the enriched nauplii was found to be a function of microalgal diet and enrichment time. The increase in the total length (TL) of nauplii enriched with both microalgae and Algamac 2000 were of equal magnitude indicating the absence of any enrichment diet specific variation in nauplii growth. Microalgal enrichment reduced the nauplii mortality. Mortality in the nauplii enriched with live microalgae was less than that of un-enriched nauplii and Algamac enriched nauplii. The study has shown that nauplii enriched with microalgae have higher tolerance to low salinity (0 ppt), when compared to the control. The high survival rate of the nauplii enriched with live microalgae will enhance their feeding by fish larvae, and reduce the deterioration of water quality in rearing tanks. Further, the salinity tolerance observed in the microalgae enriched nauplii makes them suitable for a variety of fish species under different salinity regimes.

Keywords: *Artemia, Fatty acid, Growth rate, Microalgae enrichment, Salinity stress, Survival rate*

5.2. Introduction

Aquaculture is one of the fastest growing animal food producing sectors in the world, accounting for almost half of the total food fish supply (FAO, 2010). In the present scenario, aquaculture depends on hatchery produced larvae and fingerlings than wild caught seeds. For the hatchery production of marine finfish and shellfish, availability of ideal starter diet to feed the larvae is the main concern. Most of the marine fish larvae have small mouth size and hence the larval feed should be sufficiently small during the early phase of their development (Sargent *et al.,*1997). To lessen the dependency on the natural live feed sources like *Artemia*, and to reduce the larval rearing expenses, various formulated diets have been tried as alternatives. But, due to reduced preference/acceptability by the larvae, water quality deterioration, etc. their acceptance got reduced in larviculture (Srivastava *et al.*, 2006). Among the different live feeds used in marine larviculture, *Artemia* nauplii are widely used as the starter diet. Its storage ability, easiness to hatch on demand and their soft texture make them quite essential in marine larviculture (Amat *et al.*, 2005, 2007; Abatzopoulos *et al.*, 2006; Beck and Turingan, 2007). This is in spite of the fact that they lack certain essential nutrients required for the larvae.

Among the various essential nutrients polyunsaturated fatty acids (PUFAs)*viz.*, eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3) and arachidonic acid (AA, 20:4n6) play a major role in deciding the growth and survivability of the larvae (Sorgeloos *et al.*, 1991). In nature, finfish and shellfish larvae have a limited ability to synthesize the long chain PUFAs from shorter carbon chain precursors using the desaturase and elongase enzyme mediated pathway. Hence, these are to be supplied through diets, because the lack of these essential nutrients in the diet may adversely affects the physiological functioning, survival and growth of the larvae, (Anger, 1998). Except *Artemia tibetiana*, almost all the *Artemia* species lack the long chain PUFAs, especially the 22:6n3 in its nauplii (Narciso and Morais, 2001). So, it is necessary to meet the essential PUFA requirement, through the enrichment of the above live feeds. Commercial enrichment diets such as Algamac 2000, DHA Selco, A1-Selco and, Protein Selco, etc. are widely used for this purpose (Chakraborty *et al.*, 2010; Figueiredo *et al.*, 2009; Biswas *et al.*, 2006; Sorgeloos *et al.*, 1991). However, the high PUFA content in these enrichment diets produce harmful *trans* fatty acids when exposed to light, high temperature and air which may cause larval mortalities (McEvoy *et al.*, 1995; Woollard and Indyk, 2003; Chakraborty *et al.*, 2007).

As an alternative to the commercial enrichment diets, there is a growing interest in the aqua-industry to use marine microalgae as an enrichment diet for the live feeds (Chakraborty *et al.,* 2007). Microalgae form the base of food chain and serve as the renewable reservoir of PUFAs in nature (Ferreira *et al.*, 2008). Studies on the fatty acid composition of many marine microalgae have revealed that PUFAs present in them are much more stable than many commercially available enrichment diets (Volkman *et al.*, 1989). The 22:6n3: 20:5n3: 20:4n6 ratios in live microalgal cells closely resemble natural larval diets and further, PUFAs in microalgae are better protected against oxidation by the natural antioxidants present in them. Earlier studies on microalgae revealed the importance of *Nannochloropsis*, *Chaetoceros*, and *Chlorella* as suitable enrichment diets for live feeds (Vazhappilly and Chen, 1998). The nutritional value of microalgae can vary significantly depending on the species and their culture conditions. They are amenable to mass culture and scale-up through photobioreactor and/or fermentation technology, and their nutrient profile can be optimized to a great extent by manipulating the culture conditions (Martinez-Fernandez, 2006).

The major problems associated with *Artemia* nauplii enrichment are the incidence of naupliar mortality and rapid growth during enrichment. Though the enriched nauplii have high nutrient profile, their larger size prevents them from being ingested by the small mouthed fish larvae (Sorgeloos *et al.,* 2001).

Studies on the viability of nauplii following enrichment in different media are scanty. Incidence of higher naupliar mortality before being ingested by the fish larvae remains another issue since dead nauplii are seldom preferred by fish larvae. According to Sastry (1983) larval survival rate is usually high under optimal environmental conditions, and gets reduced and finally stops when these conditions are not conducive. Since the optimum environmental conditions required for higher survival varies with the species of fish larvae, an enriched live feed able to withstand a wide range of environmental conditions will be preferable. Most of the enrichment studies on *Artemia* nauplii so far have been limited to the evaluation of the diet induced changes in the enriched nauplii, its nutrient content and growth rate (Narciso, 2000; Han *et al.*, 2001; Ritar *et al.,* 2004; Figueiredo *et al.*, 2009). However, so far, no studies have been carried out to evaluate the salinity stress tolerance potential of the post enriched *Artemia* nauplii.

The present study was aimed to

a. find out the effect of microalgae, *Pavlova viridis, Isochrysis galbana, Nannochloropsis oculata, Dicrateria inornata* and Algamac 2000, a commercial diet on the survival and growth rate of *Artemia* nauplii at different time intervals
b. find out the stress tolerance of the post enriched nauplii subjected to different saline conditions and
c. evaluate the fatty acid content of the *Artemia* nauplii enriched with microalgae *Pavlova viridis, Isochrysis galbana, Nannochloropsis oculata* and *Dicrateria inornata* at different time intervals.

5.3. Materials and methods

5.3.1. Preparation of microalgae culture for enrichment

Microalgal cultures of *P. viridis, I. galbana, N. oculata* and *D. inornata* were obtained from the marine microalgae culture facility of CMFRI, Cochin. Microalgae were cultured in Walne’s medium (Walne, 1970) in Hoffkin's glass flasks (3 L) under circadian light: dark cycle (12 h: 12 h) with a light intensity of 2000 lx at a temperature of 24 ± 1°C. Microalgae were inoculated from a stock culture (7×10^6 cells ml^{-1}) and grown up to stationary phase, and were further mass cultured with similar culture conditions. Microalgal cell density was estimated using a Neubauer haemocytometer under a microscope (Leica, Wetzlar, Germany). The cultures were maintained for one week before being used as enrichment diet for *Artemia* nauplii and further biochemical analysis. Commercial enrichment diet Algamac 2000 (0.2g. L^{-1}) was used as the control diet in the experiment after homogenizing using Ultrasonic Homogenizer.

5.3.2 Cyst hatching and enrichment of *Artemia* nauplii

Artemia franciscana cysts were collected from the hypersaline habitats of Kelambakam, Tamil Nadu (12^{0}47’ N 80^0 13’ E) during June 2010. The cysts (0.75 g L−1) were incubated (24 h) in cylindrical culture flasks (5L) holding autoclaved seawater (35‰ and 28± 1 °C) with strong bottom aeration and optimum light (2000 lx) for hatching (Sorgeloos, 1986). Metanauplii density was estimated from the subsamples (ten replicates of 1 mL subsamples). For enrichment experiment, *Artemia* nauplii were stocked (50 nauplii mL−1) in individual cylindrical tanks (5L) containing the microalgae diet (*P. viridis,I. galbana, N. oculata* and *D. inornata* respectively) (45±5 X 10^4 cells mL^{-1}) (28 ± 1^{o}C) with strong bottom aeration in triplicates (0, 1, 3, 5, 7 and 9 h of enrichment).

5.3.3 Evaluation of growth and survival rates of the enriched nauplii

Growth rates (total length) of the *Artemia* nauplii enriched with *P. viridis,I. galbana, N. oculata, D. inornata* and control diet were evaluated using the *Artemia* nauplii samples collected from each enrichment tanks randomly, and fixed in Lugol’s iodine. The total length(TL) was measured in the nauplii collected at sixdifferent time intervals (0, 1, 3, 5, 7 and 9 h of enrichment) using a light microscope (Leica, Wetzlar, Germany) attached with a camera (Leica, DFC 290) and software. Enrichment induced naupliar mortality were computed in all the enrichment experiments (microalgae and control diet) at five different time intervals (1, 3, 6 and 9 h). Nauplii survival rates were computed following the sub sampling method. The number of live nauplii in ten 1 mL subsamples in all the experiments was counted and the percentage survival of the enriched nauplii was calculated in triplicate.

5.3.4 Evaluation of salinity stress tolerance of the enriched nauplii

Salinity stress tolerance of the enriched nauplii, nine hour post enrichment (in microalgae and control diet), were estimated as follows. The enriched nauplii were transferred into cylindrical culture flasks (50 nauplii mL^{-1}) containing autoclaved water of salinities 0, 10, 20, 30 and 35 ppt., and maintained with continuous aeration with optimum temperature (28 ± $1^{o}C$) and light (2000 lx). Survival rates of the enriched nauplii at different time intervals (1, 3, 6 and 9hours) were estimated by counting the live nauplii from the subsamples (ten replicates of 1 mL subsamples).

5.3.5. Estimation of fatty acid content of the enriched nauplii

The effect of the enrichment time on the fatty acid content of the nauplii enriched was estimated from the representative samples harvested from the enrichment medium. Total lipid was extracted from both enriched nauplii and the microalgae used for the enrichment (350 mg wet weight) following the established method (Bligh and Dyer, 1959). Fatty acid composition was determined as per Metcalfe *et al.*, (1966). Triglycerides were extracted using $CHCl_3/MeOH/H_2O$ (2:4:1, v/v/v), and saponified with alkaline reagent (3 mL, 0.5 N KOH/MeOH). The saponified materials were allowed to react with a methylating mixture (14% BF_3/CH_3OH) to *Trans*-esterify the saponificable material yielding fatty acid methyl esters (FAME) that was later extracted with n-hexane/H_2O (1:2, v/v). After the removal of the aqueous layer, the *n*-hexane layer was passed through Na_2SO_4, concentrated in vacuum, reconstituted in petroleum ether (40-60^{o}C) and stored at -20°C until analysis. The esterified fatty acid content of the microalgal species and the enriched *Artemia* nauplii at different time intervals were analyzed by gas liquid chromatography with FID detector using fatty acid methyl ester standard (Supelco FAME 37 standard).

All the statistical analysis were carried out using the SPSS programme 13.0 (SPSS Inc, Chicago, USA). Means of all parameters (effect of enrichment time on growth rate, fatty acid content and survival rate) were examined for significance of the difference by analysis of variance (ANOVA).

5.4. Results

5.4.1. Effect of enrichment time on the total length of the nauplii

Irrespective of the enrichment diet, total length (TL) of the nauplii gradually increased during the nine hour enrichment period (Table 5.I) while total width gradually reduced after the study in all the experiments (Table 5.2). Total length and total width of the *Artemia* nauplii before the enrichment (zero hour) was 516.6 ± 61.4 µm and 164.6 ± 18.4 µm respectively. The nauplii length increased during the enrichment time in all the experiments including control. In the control nauplii length increased to 618.6 ± 58.6 µm after 9 hours (Fig.5.1A, V1.B, 5.C, 5.D, 5.E). Among the different enrichment dietsmaximum nauplii growth was observed in Algamac enriched nauplii (648.3 ± 70.4 µm) followed by *P.viridis* (628.3 ± 50.7 µm), *D.inornata* (623.1 ± 55.1 µm) and *N.oculata* (614.9 ± 47.3 µm). The lowest growth rate was observed in *I. galbana* enriched nauplii (600.2 ± 72.0 µm).

Table 5.1 Total length (TL) of the un-enriched, Algamac enriched and microalgae enriched *Artemia* nauplii during the nine our post enrichment.

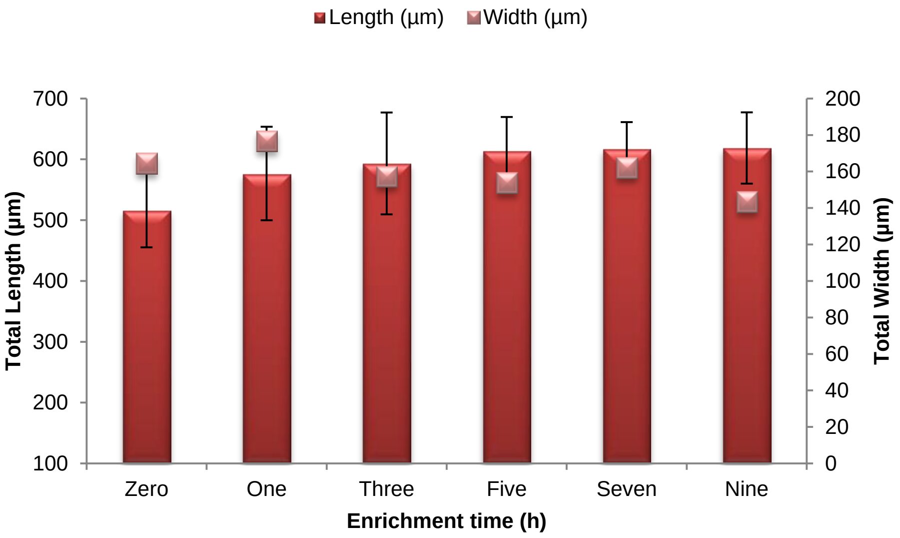
Time Hours	Un-enriched control (µm)	Algamac (µm)	*I.galabana* (µm)	*P.viridis* (µm)	*N.oculata* (µm)	*D.inornata* (µm)
0	516.6 ± 61.4	516.6 ± 61.4	516.6 ± 61.4	516.6 ± 61.4	516.6 ± 61.4	516.6 ± 61.4
1	576.6 ± 76.9	611.3 ± 82.8	560.4 ± 75.6	578.4 ± 96.3	582.1 ± 69.5	560.0 ± 68.4
3	593.2 ± 83.8	613.4 ± 77.8	575.2 ± 94.6	609.0 ± 86.2	600.0 ± 65.5	602.5 ± 59.3
5	614.3 ± 55.3	616.8 ± 59.3	593.7 ± 71.8	612.2 ± 54.7	604 ± 45.5	604.3 ± 40.0
7	617.7 ± 43.5	633.2 ± 53.3	599.2 ± 48.7	617.7 ± 37.4	606.9 ± 44.0	612.8 ± 40.3
9	618.6 ± 58.6	648.3 ± 70.4	600.2 ± 72.0	628.3 ± 50.7	614.9 ± 47.3	623.1 ± 55.1

Total width of the *Artemia* nauplii reduced gradually during the nine hour enrichment period in all the experiments (Table 5.2) including the un enriched control. In the control TW was reduced from 164.6 ± 18.47µm to 143.5 ± 12.7 after 9 hours. Similarly, in all the other experiments also TW reduced gradually after 9h enrichment the study. Lowest TW was observed in *N. oculata* enriched nauplii (142.5 ± 17.0 µm) followed by control (143.5 ± 12.7µm), *D. inornata* (151.8 ± 23.0 µm), *I. galabana* (151.9 ± 28 µm), Algamac (154.0 ± 18.3 µm) and *P. viridis* (156.0 ± 20.1 µm).

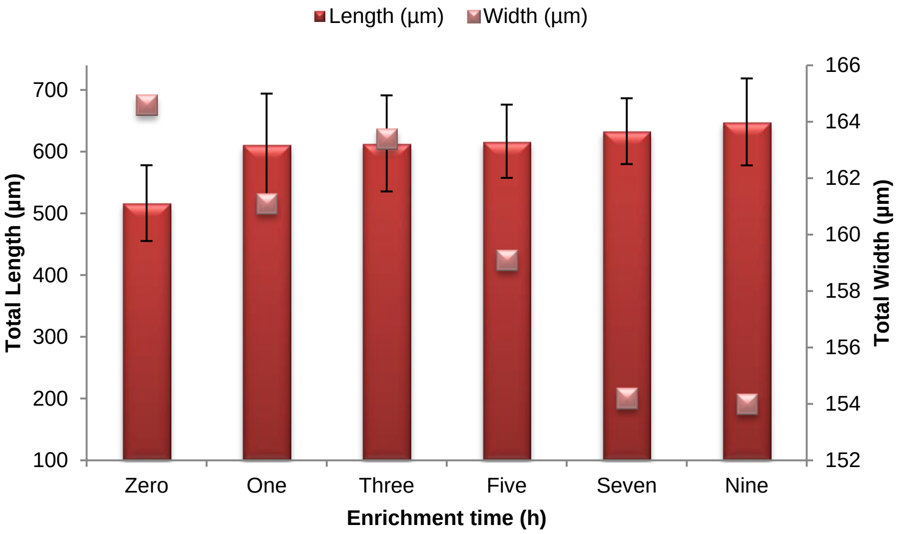
Table 5.2 Total width (TW) of the Control and enriched *Artemia* nauplii during the nine our enrichment study.

Time (h)	Control (µm)	Algamac (µm)	*I.galabana* (µm)	*P.viridis* (µm)	*N.oculata* (µm)	*D.inornata* (µm)
0	164.6 ± 18.4	164.6 ± 18.4	164.6 ± 18.4	164.6 ± 18.4	164.6 ± 18.4	164.6 ± 18.4
1	176.8 ± 11.8	161.1 ± 20.8	165.3 ± 22.1	158.3 ± 20.7	164.0 ± 16.9	159.6 ± 19.5
3	157.4 ± 17.5	163.4 ± 19.3	157.8 ± 15.8	156.9 ± 14.0	153.9 ± 20.3	165.8 ± 18.3
5	153.9 ± 17.3	159.1 ± 16.6	155.2 ± 9.07	156.9 ± 13.3	147.0 ± 9.75	161.9 ± 19.1
7	162.1 ± 17.3	154.2 ± 18.2	161.0 ± 24.6	163.4 ± 14.9	151.2 ± 13.7	153.9 ± 12.9
9	143.5 ± 12.7	154.0 ± 18.3	151.9 ± 28.1	156.0 ± 20.1	142.5 ± 17.0	151.8 ± 23.0

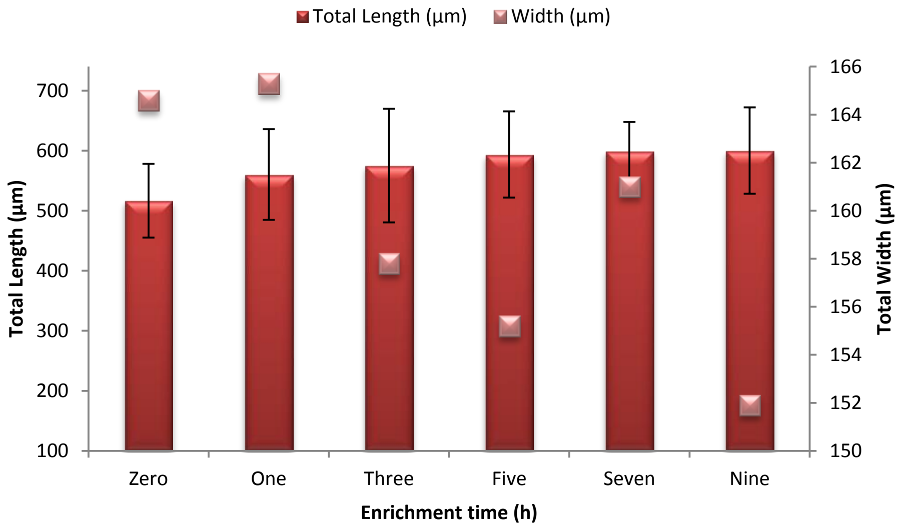
Fig. 5.1 (A) Total length and Total width of control Nauplii during the nine hour enrichment



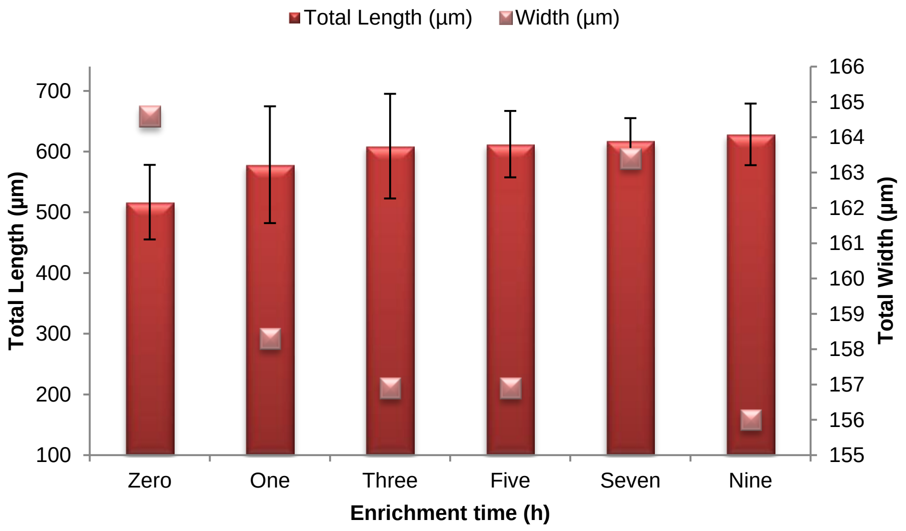
(B) Total length and Total width of Algamac enriched Nauplii during nine hour enrichment

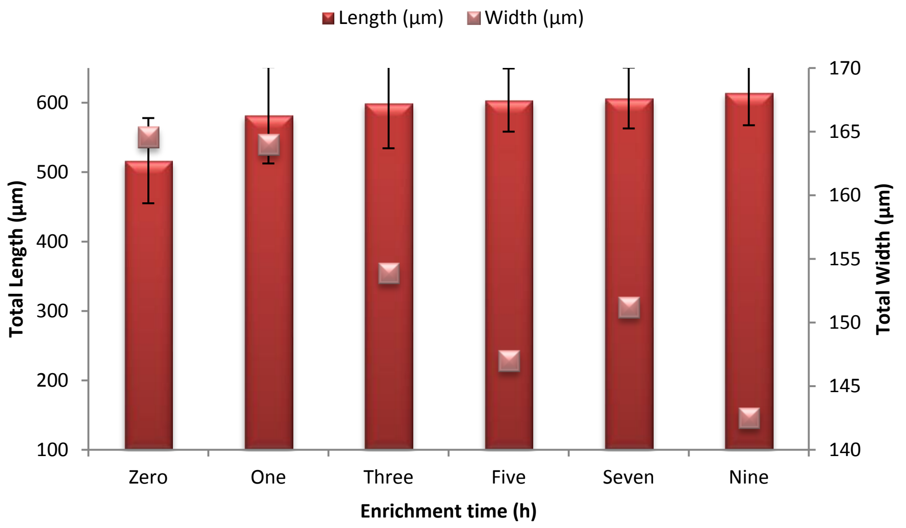


(C) Total length and Total width of *I.galbana* enriched Nauplii during nine hour enrichment

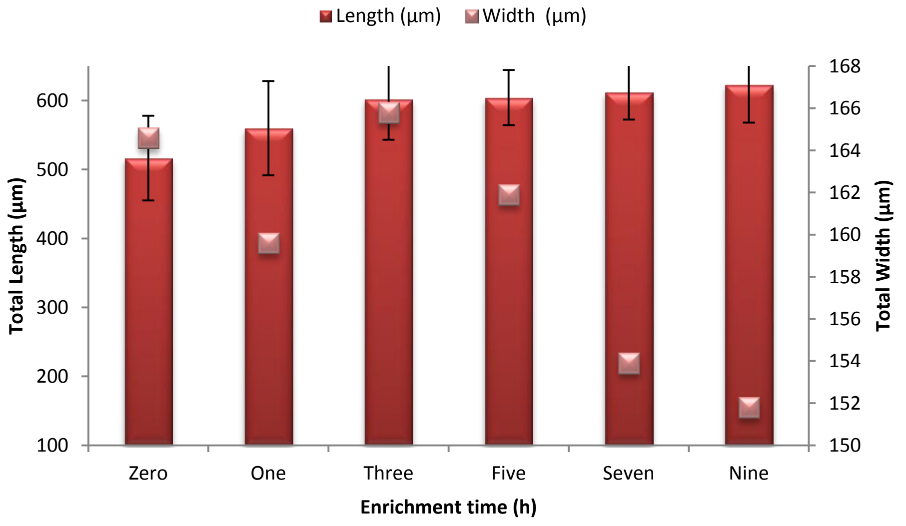


(D) Total length and Total width of *P.viridis* enriched Nauplii during nine hour enrichment





(E) Total length and Total width of *N. oculata* enriched Nauplii during nine hour enrichment



(F) Total length and Total width of *D. inornata* enriched Nauplii during nine hour enrichment

5.4.2. Mortality percentage of *Artemia* nauplii during enrichment

Significant variations in mortality percentage of *Artemia* nauplii in different enrichment diet was observed during the course of enrichment (Fig 5.2.). After nine hours of enrichment, nauplii mortality was lowest in microalgae medium than the Algamac and un-enriched/unfed medium. Mortality percentage was 18.2±5.65% in Algamac diet which was found to be significantly higher than that in microalgae viz.,*I.galbana* (3.2±1.9%), *P.viridis* (1.6±1%), *D.inornata* (0.6±0.45%) and *N.oculata* (0.5±0.37%) diet.

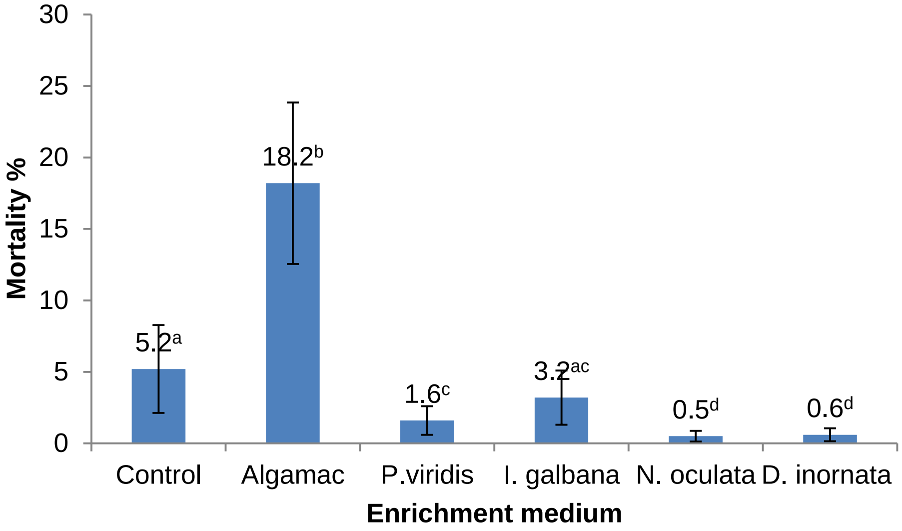


Fig.5.2 Mortality percentage of *Artemia* nauplii during enrichment

5.4.3. Salinity tolerance in enriched *Artemia* nauplii

The survival rate of the nauplii enriched for 1, 3, 6 and 9 hours with Algamac and microalgae, and the un-enriched nauplii, on exposure to 10, 20, 30 and 35 ppt salinities were 100%. However, higher mortality was observed when incubated at 0 ppt. Among the different diets, microalgae enriched *Artemia* nauplii showed significantly lower mortality percentage when compared to the

Algamac enriched and un-enriched nauplii (Table 5.3 and Fig. 5. 3). After the first 1 hour exposure to 0 ppt salinity, the Algamac enriched nauplii showed high mortality of 10.6% when compared to the un-enriched nauplii and microalgae enriched nauplii (Table 5.3). While no mortality was observed in *N. oculata* and *D. inornata* enriched nauplii, it was 1.1% in *I. galbana* and *P. viridis* enriched nauplii. At 3 hour, an increase in mortality percentage was observed in the un-enriched as well as the enriched nauplii Table 5.3. The highest mortality at 3 hours (16.3 and 16%) was observed in algamac and un-enriched nauplii. While mortality was the lowest (6.3%) in *N. oculata* enriched nauplii, it was 9% with the other three microalgae.. At 6 hour, though the mortality in the enriched group increased, it was lower than the control and Algamac enriched nauplii. The lowest mortality was in the *N. oculata* and *D. inornata* enriched nauplii (9.7%) followed by *I. galbana* and *P. viridis* enriched nauplii (10.4 and 11%) as compared to the 16.7% mortality in Algamac enriched and un-enriched nauplii (16.7%). After the 9 hour incubation at 0 ppt, highest mortality was observed in unenriched nauplii (25.2%) followed by algamac enriched nauplii (19.4%) when compared to the microalgae enriched nauplii which ranged from 10.6 to 13.1% only (Table 5.3). Among all the enrichments, *I. galbana* resulted in the lowest mortality percentage after holding at 0 ppt. salinity for 9 hours.

Table 5.3 Mortality Percentage of un-enriched control, Algamac enriched and microalgae enriched *Artemia* nauplii incubated at 0 ppt.,for different durations.

Incubation Time	Un-enriched	Algamac	I.galbana	P.viridis	N.oculata	D.inornata
1 hour	0.6	10.6	1.1	1.1	0	0
3 hour	16	16.3	9	9	6.3	9
6 hour	16.7	16.7	10.4	11	9.7	9.7
9 hour	25.2	19.4	10.6	12.2	13.1	11.5

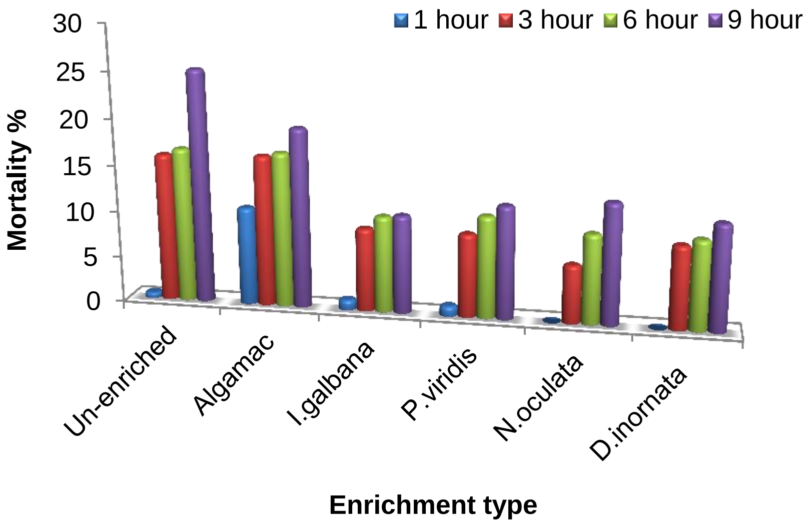


Fig. 5.3. Mortality percentage of un-enriched control, Algamac enriched and microalgae enriched *Artemia* nauplii incubated at 0 ppt., for different durations.

5.4.4. Effect of enrichment time on the fatty acid profile of the nauplii

Fatty acid profile of the different microalgae used for enrichment was worked out as a pre-mediated step, and it has revealed notable differences among them. Fatty acid profile of the microalgae showed considerable variations in the level of major fatty acids. Saturated fatty acids (SFAs) were found to be low in the microalgae, *D. inornata* and *I. galbana* (23.81 and 28.95%, respectively) compared to *P. viridis* and *N. oculata* (35.16 and 38.64 %, respectively) (Table 5.4a, 5.4b & Fig 5.3).

Impact of enrichment time and microalgal species on the fatty acid profile of the *Artemia* nauplii are illustrated under Table 5.4a &5.4b. Though, a gradual decline in SFAs was observed in all the microalgae enriched nauplii up to 7th hour of enrichment, it was only marginal. However, after 9th hour of enrichment, the SFA content of the nauplii showed a significant increase. Among the different SFAs, fatty acid14:0 and 16:0 contributed the major share in the nauplii. Interestingly, the 9 hour enriched nauplii showed a significant positive correlation between the SFA in it and the microalgal SFA, while no correlation existed in others.

Table 5.4a. Fatty acid composition of microalgae (*P. viridis* and *D. inornata*) *vis-à-vis Artemia* nauplii enriched with microalgae.

	P.viridis	***Artemia nauplii enriched with P.viridis***						***D.inornata***	***Artemia nauplii enriched with D.inornata***					
Duration		**0**	**1**	**3**	**5**	**7**	**9 h**		**0**	**1**	**3**	**5**	**7**	**9 h**
Component							**Saturated**	**Fatty Acids**						
12:0	0.29	0.08	0.06	0.05	0.03	0.03	1.38	0.93	0.08	0.06	0.06	0.05	0.07	0.88
13:0	0.04	0.03	ND[a]	ND	ND	ND	0.27	0.62	0.03	0.03	0.01	ND	ND	0.25
14:0	11.25	3.53	3.49	3.38	2.74	2.65	5.09	1.35	3.53	3.26	2.59	3.15	3.8	6.92
15:0	0.86	1.39	1.34	1.22	0.41	0.38	0.85	0.59	1.39	1.28	0.57	0.52	0.48	1.24
16:0	19.37	19.3	19.2	18.7	18.3	17.6	22.5	18.91	19.3	19.1	18.6	18.2	18.2	23.4
17:0	0.14	1.52	1.48	1.35	1.28	1.19	1.53	0.10	1.52	1.38	1.29	1.25	0.51	0.84
18:0	1.92	5.1	5.04	4.85	4.24	4.18	7.27	0.48	5.1	5.29	5.64	5.87	4.55	5.06
20:0	0.11	0.04	ND	ND	ND	ND	0.08	0.08	0.04	0.29	0.55	0.73	0.56	0.82
22:0	0.03	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.08
24:0	1.15	0.12	0.09	0.06	0.05	0.03	0.15	0.75	0.12	0.64	1.07	1.38	0.98	1.21
ΣSFA	35.16	31.1	30.7	29.7	27.1	26	39.1	23.81	31.1	31.3	30.4	31.1	29.2	40.7
							Monounsaturated	**Fatty Acids**						
14:1n9	0.38	0.03	ND	ND	0.03	0.05	0.06	0.23	0.03	0.03	ND	ND	ND	ND
16:1n7	27.9	13.6	13.5	12	12	10.1	9.32	0.64	13.6	12.6	11.5	8.82	7.5	7.19
16:1n9	0.52	0.06	0.04	ND	ND	ND	ND	8.16	0.06	0.05	0.03	0.03	0.03	0.03
18:1n7	1.64	0.12	0.06	0.03	0.03	ND	ND	0.13	0.12	0.1	0.08	0.06	0.05	0.05
18:1n9	7.41	28.5	28.4	27.5	26.3	24.6	21.5	23.57	28.5	27.7	25.1	24.1	22.5	22.1
24:0	0.86	0.08	0.05	0.03	ND	ND	ND	0.34	0.08	0.06	0.05	0.03	0.03	0.12
ΣMUFA	38.71	42.4	42.1	39.6	38.3	34.8	30.9	33.07	42.4	40.6	36.8	33.1	30.2	29.4
							Polyunsaturated	**fatty Acids**						
18:2n6 cis	3.48	7.52	8.2	8.61	8.74	8.92	8.82	6.3	7.52	9.83	10.4	10.6	10.8	8.56
18:3n6	1.05	1.18	1.29	1.67	2.24	2.68	0.53	0.39	1.18	1.35	1.42	1.54	1.68	1.19
18:4n6	0.72	0.03	0.09	0.16	0.22	0.62	0.46	0.14	0.03	ND	ND	ND	ND	ND
18:3n3	1.47	3.21	3.82	3.91	4.19	4.26	3.09	12.84	3.21	3.53	5.92	5.89	6.72	5.26
18:4n3	2.9	0.08	0.35	0.49	0.58	0.87	0.73	8.65	0.08	0.2	1.17	1.35	2.26	1.93
C20:2n6	0.24	0.15	0.19	0.21	0.16	0.25	0.18	0.19	0.15	0.23	0.45	0.57	0.65	0.41
C20:3n6	0.18	1.1	1.23	1.34	1.48	1.70	1.15	1.06	1.1	1.35	1.37	1.51	1.93	1.28
C20:4n6	1.86	1.35	1.14	1.62	2.07	2.31	1.82	0.98	1.35	1.42	1.61	1.73	1.93	0.85
20:5n3	9.54	3.08	3.26	3.79	4.15	7.58	4.12	6.82	3.08	3.12	3.19	4.18	5.98	3.06
22:5n3	0.16	0.02	0.04	0.06	0.09	0.48	0.32	0.38	0.02	ND	0.03	0.12	0.08	0.06
22:6n3	1.81	0.32	0.43	1.48	2.72	1.37	0.85	1.26	0.32	0.36	1.09	1.95	1.85	0.82
ΣPUFA	23.41	18	20	23.3	26.6	31	22.1	39.01	18	21.4	26.6	29.4	33.9	23.4
∑ n3	15.88	6.71	7.9	9.73	11.7	14.6	9.11	29.95	6.71	7.21	11.4	13.5	16.9	11.1
∑ n6	7.53	11.3	12.1	13.6	14.9	16.5	13	9.06	11.3	14.2	15.2	15.9	17	12.3

n3/n6	2.11	0.59	0.65	0.71	0.79	0.88	0.7	3.31	0.59	0.51	0.75	0.85	0.99	0.91
∑ PUFA/∑ SFA	0.67	0.58	0.65	0.79	0.98	1.19	0.56	1.64	0.58	0.68	0.88	0.94	1.16	0.58
DHA/EPA	0.19	0.10	0.13	0.39	0.65	0.18	0.21	0.18	0.10	0.12	0.34	0.47	0.31	0.27

The individual fatty acid is expressed as the percentage of total fatty acids. [a]ND, fatty acid identified on GC trace but not integrated by the instrument. ΣSFA, total SFAs; ΣMUFA, total MUFAs; and ΣPUFA, total PUFAs

Table 5.4b. Percentage fatty acid composition of microalgae (*N. oculata* and *I. galabana*) *vis-à-vis Artemia* nauplii enriched with microalgae for up to 9 hour enrichment

	N.oculata	*Artemia nauplii enriched with N.oculata*						*I.galabana*	*Artemia nauplii enriched with I.galabana*					
Duration		**0**	**1**	**3**	**5**	**7**	**9 h**		**0**	**1**	**3**	**5**	**7**	**9 h**
Component	**Saturated Fatty Acids**													
12:0	0.94	0.08	0.06	0.05	0.05	0.03	2.6	0.56	0.08	0.03	ND	ND	ND	0.13
13:0	0.29	0.03	ND	ND	ND	ND	0.12	0.08	0.03	ND	ND	ND	ND	0.05
14:0	5.19	3.53	3.32	3.2	3.15	3.2	5.14	6.35	3.53	3.41	3.34	3.35	2.68	5.14
15:0	1.07	1.39	1.33	1.2	1.18	1.73	2.16	0.81	1.39	1.36	1.27	1.23	0.91	1.73
16:0	21.59	19.3	19.2	19.1	18.8	20.6	27.2	14.5	19.3	19.2	18.3	18.1	17.8	26.2
17:0	0.09	1.52	1.48	1.48	1.42	1.36	3.94	0.03	1.52	1.5	1.48	1.47	1.25	1.86
18:0	8.76	5.1	4.47	4.51	4.43	3.65	9.84	5.93	5.1	5.06	4.89	4.74	4.5	6.38
20:0	0.11	0.04	0.05	0.03	ND	ND	0.63	0.05	0.04	ND	ND	ND	0.03	0.08
22:0	0.06	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24:0	0.54	0.12	0.1	0.08	0.06	0.05	0.17	0.64	0.12	0.08	0.05	0.05	0.06	0.18
ΣSFA	38.64	31.1	30	29.7	29.1	30.7	51.8	28.95	31.1	30.7	29.3	28.9	27.2	41.7
Monounsaturated Fatty Acids														
14:1n9	0.08	0.03	0.03	0.02	ND	ND	0.03	0.19	0.03	ND	ND	ND	0.05	ND
16:1n7	16.88	13.6	13.2	12.7	12	12	8.3	3.59	13.6	13.5	13.2	11.5	10.9	9.24
16:1n9	0.13	0.06	0.04	0.03	ND	ND	ND	0.15	0.06	0.04	0.04	0.03	ND	ND
18:1n7	0.14	0.12	0.09	0.06	0.04	0.04	0.11	0.08	0.12	0.08	0.05	0.05	0.03	0.03
18:1n9	19.5	28.5	27.9	27.3	25.1	24.3	21.2	20.25	28.5	28.3	28.2	26.1	23.6	21.6
17:01	0.05	ND	ND	0.08	0.05	0.03	0.07	ND	ND	ND	ND	ND	ND	ND
24:00	1.23	0.08	0.06	0.04	0.09	0.06	0.19	0.28	0.08	0.06	0.08	0.05	0.03	0.03
ΣMUFA	38.01	42.4	41.3	40.2	37.3	36.4	29.9	24.54	42.4	42	41.6	37.7	34.6	30.9
Polyunsaturated Fatty Acids														
18:2n6 cis	4.63	7.52	7.69	8.13	8.75	8.83	4.15	8.48	7.52	8.14	9.09	10	10.6	6.27
18:3n6	0.35	1.18	1.23	1.38	1.56	1.43	0.64	0.15	1.18	1.15	1.21	1.39	1.45	0.51
18:4n6	0.04	0.03	ND	ND	0.05	0.02	ND	0.04	0.03	0	0.15	0.18	0.64	0.47
18:3n3	0.67	3.21	3.38	3.42	3.69	3.5	1.92	5.78	3.21	3.25	3.56	4.18	4.54	4.05
18:4n3	0.15	0.08	0.15	0.18	0.23	0.14	0.09	15.32	0.08	0.13	0.21	0.26	0.34	0.28
C20:2n6	0.82	0.15	0.21	0.29	0.36	0.25	0.18	0.08	0.15	0.18	0.21	0.2	0.39	0.25

C20:3n6	0.54	1.1	1.24	1.72	1.76	1.72	0.18	0.19	1.1	0.98	1.15	1.24	1.32	0.86
C20:4n6	2.15	1.35	1.42	1.86	2.15	1.72	1.51	0.48	1.35	1.42	1.59	1.68	2.21	1.39
20:5n3	9.69	3.08	4.87	5.09	7.81	6.93	3.63	2.60	3.08	3.27	3.58	4.05	4.11	3.03
22:5n3	0.13	0.02	ND	0.06	0.06	0.03	ND	0.44	0.02	ND	ND	0.03	0.05	ND
22:6n3	0.64	0.32	0.12	0.11	1.22	1.15	0.62	9.75	0.32	0.28	0.69	1.38	3.69	1.41
ΣPUFA	19.81	18	20.3	22.2	27.6	25.7	12.9	43.31	18	18.8	21.4	24.6	29.3	18.5
∑ n3	11.28	6.71	8.52	8.86	13	11.8	6.26	33.89	6.71	6.93	8.04	9.9	12.7	8.77
∑ n6	8.53	11.3	11.8	13.4	14.6	14	6.66	9.42	11.3	11.9	13.4	14.7	16.6	9.75
n3/n6	1.32	0.59	0.72	0.66	0.89	0.84	0.94	3.60	0.59	0.58	0.6	0.67	0.77	0.90
∑ PUFA/∑ SFA	0.51	0.58	0.68	0.75	0.95	0.84	0.25	1.50	0.58	0.61	0.73	0.85	1.08	0.44
DHA/EPA	0.07	0.1	0.02	0.02	0.16	0.17	0.17	3.75	0.1	0.09	0.19	0.34	0.9	0.47

The monounsaturated fatty acid (MUFA) content in nauplii enriched with *P. viridis, D. inornata, N.oculata* and *I.galabana* showed a gradual reduction after nine hours of enrichment (30.9%, 30.2%, 29.9% & 30.9% respectively), when compared to the unenriched nauplii (42.4%). No correlation was observed between the MUFA content in the microalgal diet and the enriched nauplii. Total MUFA of the enriched nauplii is contributed mainly by 18:1n9 (60%) followed by 16:1n 7 (30%).

PUFA content of the microalgae sources used for enrichment revealed wide variation. Among the microalgae sources evaluated, *I. galbana* showed the highest PUFA content (43.31%) followed by *D. inornata* (39.01%), *P. viridis* (23.41%) and *N. oculata* (19.81%). In the nauplii enriched with these microalgae, time dependent increase in the total PUFA content was clear though,the variations were only marginal(Table 5.4a & 5.4b).

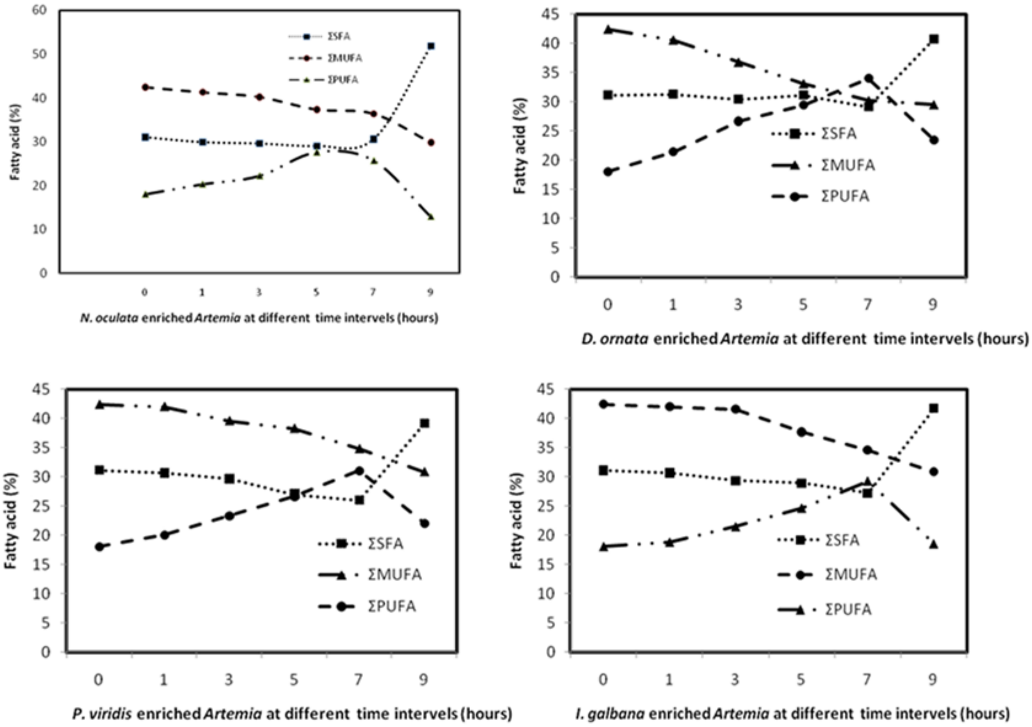


Fig.5.4 SFA, MUFA and PUFA content of *Artemia* nauplii enriched with four microalgae

The differential composition of ∑PUFA (Total polyunsaturated fatty acid), ∑SFA (Total monounsaturated fatty acid) and, ∑MUFA (Total monounsaturated fatty acids) *Artemia* nauplii enriched with microalgae at different enrichment intervals.

The maximum total PUFA level was reached by 7 hours following enrichment in *I. galbana, P. viridis* and *D. inornata* respectively and it was 29.3, 31 and 33.9% respectively. However, by 9th hour it got reduced considerably to 18.5, 22.1 and 23.4% respectively. On the other hand the total PUFA in the nauplii enriched with *N. Oculata* reached the maximum at the 5th hour post enrichment (27.6 %).However, it also started to decline after the 7thhour(25.7%), and by 9th hour it declined to 12.9 % (Table 5.4a& 5.4b). The 18:2n6, 20:4n6 and 20:5n3 contributed the major share of total PUFA in the different microalgae diet used for the enrichment study. While 18:3n3 and 20:5n3 were high at 7th hour of enrichment with *D. inornata* (6.72% and

5.98%), *I. galbana* (4.54 and 4.11%) and *P. viridis* (4.26 and 7.58%), in case of *N. oculata* they reached the maximum level at 5th hour of enrichment(3.69 and 7.81%).The maximum level of 20:5n3 with *N. oculata*(7.81%) and with *P.viridis* (7.58) were comparable, but in case of the former it was reached in 5 hours as against 7 hours in the later. In case of 20:4n6 and 18:3n6 an incremental trend was visible wherein the maximum level was reached at 5th hour of enrichment with *N. oculata* (2.15 and 1.56% respectively), while in all others maximum was reached after 7th hour of enrichment. Total n3 PUFAs were high in *N. oculata* enriched nauplii, and it ranged from 8.52 (during 1st hour of enrichment) to 13% (during 5th hour of enrichment) (Fig. 5.4).

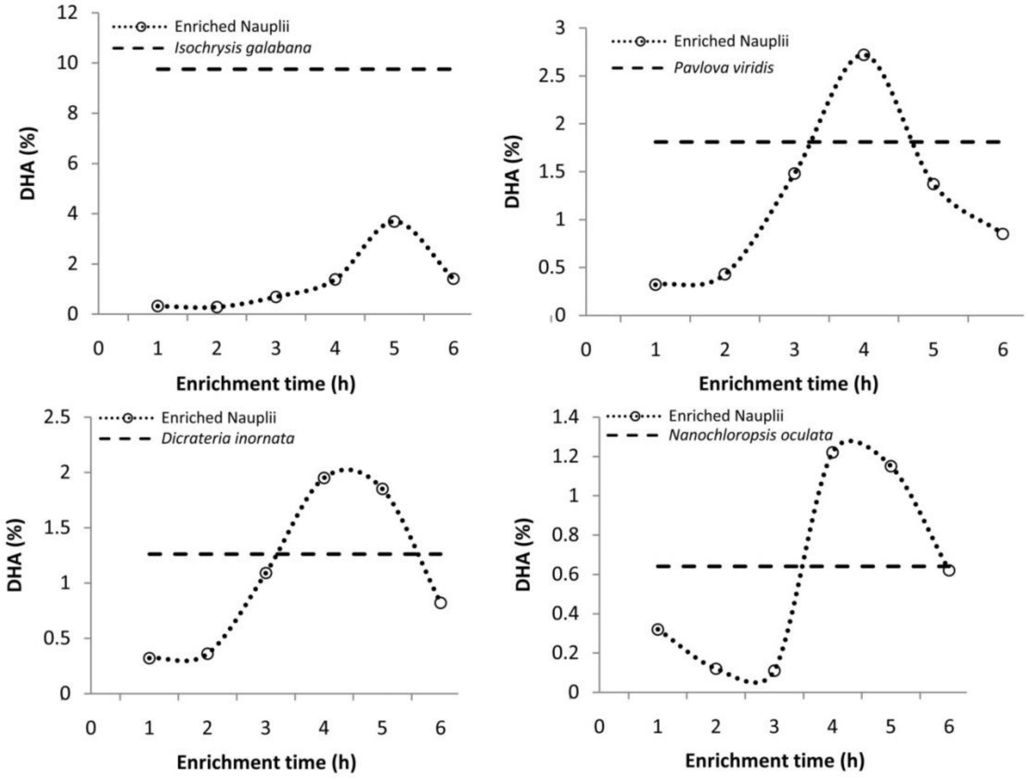


Fig. 5.5. DHA (22:6n 3) content in microalgae and the enriched *Artemia* nauplii

5.5. Discussion

Prey size has always been a key limiting factor influencing the feeding efficiency of the predatory larvae. de Barros and Valenti, (2003) have suggested that the most favorable relationship of prey size to predator length is 0.2, indicating the importance of small size prey in larviculture. In the present study it was seen that during the nine hours of enrichment, there was a gradual increase in the total length (TL) of the nauplii,be it the un-enriched control or enriched with microalgae or Algamac, indicating the absence of any enrichment diet specific variation in nauplii growth. The total width of the nauplii got reduced gradually during the nine hours of study in the control nauplii as well as the nauplii enriched with various microalgae and Algamac due to the usage of yolk sac for initial growth.

One of the major drawbacks associated with the enrichment of *Artemia* nauplii is the reduction in naupliar survival rates. However, the present study has indicated high survival rate following enrichment with microalgae in comparison to the Algamac. Among the four microalgae, *N.oculata* and *D. inornata* showed very low mortality percentage as compared to the *P.viridis* and *I. galbana*. During the nine hour period, considerably high mortality was detected in the un-enriched nauplii also.

Low nauplii mortalities percentage in microalgae enrichment than the un-enriched control and Algamac enrichment could be attributed to the effect of biomolecules such as betaine, inosine 5-monophosphate and free amino acids present in the microalgae as suggested by Muller-feuga, (2000) and Kolkovski *et al.,* (1997). High nauplii mortality on enrichment with Algamac 2000has been reported by Figueiredo *et al.,* (2009) from a time course enrichment study.

Superiority of a live feed in larviculture depends to a great extent on their versatility to acclimatize to sudden environmental changes when added to the larval rearing tanks. Major environmental factors which limit the development and survival of larvae in aquaculture include salinity, temperature, pH, etc., their specific requirement varying with the species reared (Paul and Paul, 1999). Among these environmental parameters, in case of mariculture, salinity stands out as the most important factor. Therefore, the salinity tolerance of a live feed a make it ideal for the larviculture of marine species. The present study have shown that nauplii enriched with microalgae have higher tolerance to low salinity especially to0 ppt, indicating their better osmotic stress tolerance when compared to the control diet enriched nauplii. Fish larvae always prefer to ingest moving or swimming nauplii, and the higher percentage of dead nauplii in larval culture tanks will result in reduced feeding leading to water quality problems and disease outbreaks. Thus, the higher tolerance to saline conditions observed in this study is a plus point in using microalgae enriched nauplii as live feed in larval rearing. It could be presumed that the very long-chain fatty acids (VLCFA) assimilated from the microalgae have played a role in protecting the membrane integrity, and thereby raising the salinity tolerance, following the report of Prasitchoke *et al.,* (2007) that VLCFAs are important for membrane construction and stabilization in eukaryotic cells. Though, the commercial enrichment medium is also rich in VLCFAs, high mortality rate was observed when *Artemia* was kept in this medium for enrichment. It was also observed that few of the *Artemia* nauplii became less active and immobile. It is to be deduced that the enrichment medium weakens the nauplii reducing their ability to live in saline conditions. The enhanced salinity tolerance of the microalgae enriched nauplii can improve the feeding efficiency of the larvae, and retain the water quality conditions in the larval rearing systems, especially in low saline conditions.

Fatty acids, especially the polyunsaturated fatty acids (PUFAs) have a vital role in deciding the survival, growth and development of finfish larvae. The long chain PUFAs *viz.*, 20:5n3 and 20:4n6 are mostly involved in the production and modulation of eicosanoids (Brown, 1994), while the 22:6n3 function to keep the structural and functional integrity in larval cell membranes, including neural function (Chakraborty *et al.,* 2007; Bell *et al.,* 2003b). However, finfish larvae have a very limited ability to synthesize the PUFAsin required quantities. Freshly hatched nauplii, though, had high level of 20:5n3 (3.08%) showed low level of 20:4n6 (1.35%) and very low level of 22:6n3 (0.32%).This being the general trend as reported by several authors (Han *et al.*, 2001; Narciso and Morais, 2001, Kara *et al.,* 2004), calls for the supplementation of the nauplii with essential fatty acids before feeding the larvae (Sargent *et al.,* 2002; Bell *et al.,* 2003a; Monroig *et al.,* 2006).

In view of some of the draw backs of the commercial enrichment diets, the present study was undertaken to evaluate the advantages of using marine microalgae as an alternative. A pre-mediated evaluation of the fatty acid composition of the microalgae used for enrichment trials have given interesting results. Though, only small amounts of C_{20} fatty acids were present in *I. galbana* and *D. inornata*, the precursors for their biosynthesis, namely C_{18} PUFAs 18:2n6 cis and 18:4n3 were present in large amounts. Among all the microalgal dietary sources studied, *I. galbana* had high level of 22:6n3, C_{18} fatty acids and total PUFA, followed by *D. inornata* and *P. viridis*.

Fatty acid content in the enriched nauplii varied as a function of microalgae used and enrichment time. Essential fatty acids *viz.,* 20:5n3 and

22:6n3 increased with the progress of enrichment up to 5-7 hours of post enrichment. Nauplii enriched with *I. galbana* for seven hours had the highest 22:6n3 content (3.69%), followed by *P. viridis* (2.7%). In case of enrichment with *D. inornata* the maximum level(2%) was attained even faster viz., after five hours of enrichment (Fig 5.5). The 22:6n3 content of the enriched *Artemia* nauplii during the present study was far higher than that of *Artemia* nauplii enriched with a mixture of *Nannochloropsis salina, Chaetoceros calcitrans,* and *Chlorella salina* reported by Chakraborty *et al.,* (2007). However, Figueiredo *et al.,* (2009) have reported 6.45% of 22:6n3 in *Artemia* nauplii enriched with Algamac 2000 which has a DHA content of 27% showing an assimilation of 23.88%. The present study shows that *Artemia* nauplii enriched with live microalgae with an average DHA content of 3.37% has succeeded in assimilating an average 2.01% of 22:6n3, indicating a far higher percentage of 22:6n3 assimilation. The present study shows that among the microalgae evaluated, *N. oculata* had the highest content of 20:5n3. Nauplii enriched with*N. oculata* yielded notable level of 20:5n3 at 5th hour namely, 7.81%. However, in case of *I. galbana* the comparable 20:5n3 level (4.11%) was reached at 7th hour of enrichment. These values are higher than the values reported from *Artemia salina* nauplii enriched with baker's yeast and microalgae (4.2%), and *A. franciscana* nauplii enriched with *Odonus niger* liver oil (2.5 to 5.1%) (Chakraborty *et al.,* 2007 and Immanuel *et al.,* 2007), suggesting the superiority of *N. oculata* and *I. galbana* as enrichment diets. The drop in 20:5n3 after the 7th hour of enrichment may be due to inadequate rate of desaturation (by Δ5-fatty acid desaturase) and elongation of 18:3n3 or 18:4n3 (Chakraborty *et al.,* 2007). The higher level of 20:5n3 (2.6%)and 22:6n3 (9.75%) in *I. galbana* is also reflected in the nauplii after the enrichment indicating theirability to effectively amass enough PUFAs in nauplii.

The total PUFA content of the nauplii enriched with live microalgae reached maximum by the 7th hour of enrichment (except in *N. oculata* enriched nauplii) followed by a significant drop by the 9th hour. The linolenic acid (18:3n−3) constituted a very low percent of the total PUFA in all the microalgae(3.38 and 13.34 %) except *D. inornata*. This is suggestive of the nutritional superiority of live microalgae as an enrichment diet, because according to Buzzi *et al.,* (1996) low linolenic acid (18:3n−3) in enrichment diets can increase the bioconversion of 20:5n3 to 22:6n3. This study has shown that short time enrichment (5 h) with *N. oculata* can improve the PUFA profile of the nauplii while a minimum of seven hour is required when using the *I. galbana, D. inornata* and *P. viridis* species.

Selected live microalgae or their combinations can be used as excellent enrichment dietary sources for *Artemia* nauplii, which in turn can provide many of the vital nutrients essential for the fish larvae in larviculture. The present study reveals that five to seven hour of enrichment of *Artemia* nauplii with live microalgae diets can significantly improve the essential PUFA content while keeping the nauplii size at minimum. The high survival rate of the nauplii enriched with live algae will enhance the feeding of the fish larvae and reduce the deterioration of water quality in the rearing tanks. Further, the high tolerance to low salinity makes them suitable for being used as live feed for a variety of fish species under different salinity regimes.

5.6. Conclusions

Present study explored the suitability of using live microalgae as enrichment diet for *Artemia* nauplii. Microalgae are superior enrichment diet for *Artemia* nauplii due to the higher survival rate, nutritional content and salinity tolerance in the enriched nauplii as compared to the commercial enrichment diet.

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Chapter 6

Differential expression of biochemical constituents and real time analysis of ArHsp22 gene in *Artemia* under salt stress

Vikas P.A, Sajeshkumar N.K, KajalChakraborty, Sanil N.K, Thomas P.C and Vijayan K.K.

Analysis of the genetic and biochemical adaptations of Arthropods to withstand salt stress in hypersaline habitats; A case study. Communicated

6.1. Abstract

The brine shrimp, *Artemia* is distributed along the hypersaline inland salt lakes and salt pans, and are rarely found in waters with salinity less than 45 ppt., although physiologically they can survive in seawater. Salinity, no doubt is the predominant abiotic factor which determines the presence of *Artemia* in a water body, and consequently limits its geographical distribution. The present study was carried out to elucidate the effect of salinity on the biochemical constituents of *Artemia*. Indigenous *Artemia sp.* Were exposed to salinities of 100, 150 and 200 ppt for 24 hours and the biochemical constituents such as soluble proteins, amino acids, trehalose and fatty acids, the expression of heat shock protein gene ArHsp 22 and the survival rate at 6th and 24th hour of exposure were estimated. The present study has shown that *Artemia* adults can tolerate sharp increase in salinity up to 150 ppt with low mortality rates, but increases in salinity beyond 200ppt induces higher mortalities. The level of soluble protein in *Artemia* maintained at 150 and 200 ppt showed a significant reduction up to 6 hours of incubation. However, after 24hours of incubation the protein level of 150 pp. increased and returned back to the normal levels, while at 200 ppt. the protein level have shown a further reduction. Short term (6 hr.) exposure of *Artemia* to salinity stress resulted in a significant increase in total AA content. It continued to increase significantly during long term exposure (24hour) to salt stress, except in the case of 200ppt. Though, carotenoid level showed a reduction at 6 hours, it started to increase as the exposure time prolonged and by 24 hours reached very high levels at 100 and 150 ppt.. In case of trehalose, the initial response was a sharp increase in the level, and at 6 hours level was substantially high. However, by 24th hour the trehalose levels decreased sharply, but were still higher than the controls. Following 6 hours of exposure to salinity stress, the total PUFA increased significantly at 100 ppt. and 150 ppt. while at 200 ppt. it was reduced. However after 24 hours of exposure, the PUFA levels reduced sharply at 100 ppt, remained at the same level at 150 ppt and significantly increased at 200 ppt.. Among the various components of PUFA, the level of 22: 6n 3 was significantly high at all salinities at 6 and 24 hours of incubation when compared to the control. It remained high and stable at 150 ppt. at 6 and 24 hours of incubation. Expression of ArHsp 22 gene increased marginally at 100 ppt. while increased sharply at 150 and 200 ppt after 6 hours of incubation. Present study conclude that *Artemia* can withstand sudden increase in salinity up to 150 ppt without any mortality up to 6 hours of durations and with low percent mortality up to 24 h incubation. Enhanced expression of constituents such as proteins, amino acids, trehalose, carotenoids, fatty acids and ArHsp 22 in *Artemia* might have acted as the first line of defense against the sudden salinity stress in *Artemia*.

Keywords: *Salt stress, Trehalose, Carotenoid, Fatty acid, Amino acid, ArHsp22, Soluble protein, Survival, Gene Expression*

6.2. Introduction and review of literature

The ecosystems are always in a dynamic state and there is a nature defined limits where the animals can live naturally. Animals possess different physiological and morphological adaptations to thrive in the harsh environments of nature. Aquatic animals are more susceptible to sudden variations in the living medium than the terrestrial animals due to the necessity of osmotic balance to live in the aquatic ecosystem. Both the marine and fresh water organisms possess different mechanisms to overcome the varying environmental conditions. Physical and biological conditions of the ecosystems being in a dynamic state, nature has set limits within which the animals can live naturally. The state at which the physical and biological conditions exceed the dynamic equilibrium of the ecosystem often leads to the destruction of fauna flora relations in it. Seley, (1936) coined this state as “stress” after observing the influence of external stimuli called "noxious agents" to cause imbalance or symptom to the organism. Stress in other words can be defined as a specific condition where the innate dynamic equilibrium of the nature and homeostasis of animals is threatened or disturbed due to the differential actions of intrinsic or extrinsic stimuli, commonly defined as stressors (Chrousos and Gold, 1992; Wendelaar Bonga, 1997).

In aquatic environments, organisms experience different stressors mainly due to the anthropogenic interventions, particularly the burning of fossil fuels which drastically alter the global climate (Cavallo *et al.*, 2010; Barton and Iwama, 1991), and also due to the natural stressors which are formed either as the end effect of anthropogenic interventions or as a result of natural calamities (Kelly *et al.*, 2000). According to the Intergovernmental Panel on Climate Change (IPCC), (2007) “Warming of the climate system is unequivocal, as is now evident from observations of increases in global average air and ocean temperatures, widespread melting of snow and ice, and rising global average sea level". Temperature shifting and salinization of freshwater bodies and hypersalinazation of saline ecosystems are the first and immediate stressors which directly cause an imbalance of the ecosystem (Cavallo *et al.*, 2010; Krishnakumar *et al.*, 1991). Since the aquatic organism are in close contact with the medium via their gills, exoskeleton, skin and scales, their stress response are swifter than the terrestrial animals. This in turn, negatively affect the animal homeostatic equilibrium, feed conversion ratio (FCR), specific growth rate (SGR), survival, reproductive performance and immune system (Anger 2003; Wendelaar Bonga, 1997).

To conquer the hyper salinization effects in aquatic ecosystems, the aquatic fauna and flora adapt different mechanisms depending on the species/ecosystem. According to Kültz, (2007), during stress, the animals sense and quantify the changes, mainly the environmental and extracellular osmolality variations, and initiate the compensatory and counteracting effector mechanisms by signal transduction of information about osmolality changes from osmosensors.

Brine shrimp, *Artemia* are distributed along the hypersaline water bodies, inland salt lakes and salt pans around the globe. They are rarely found in waters with salinity lower than 45 ppt, although, physiologically they thrive in seawater and even in brackish waters (Persoone *et al*., 1980). Osmotic pressure and ionic composition of *Artemia* haemolymph vary significantly with the medium and even in the concentrated medium their haemolymph osmotic pressure would be always lesser than marine invertebrates living in normal sea water. And this level is similar to that of a normal fresh-water animal with high Na:Cl ratio and low magnesium concentration. The above physiological indicates points out to a fresh-water ancestry for the *Artemia* sp. (Croghan

1957). Salinity is no doubt the predominant abiotic factor determining the existence of *Artemia* in nature, and also act as a natural defense mechanism against predators/competitors, and consequently, limiting its geographical distribution (Steppen, 2002). Studies by Triantaphyllidis *et al.,* (1995), El-Bermawi *et al.,* (2004) and Agh *et al*., (2008) showed that the growth rate of *Artemia* is inversely proportional to the salinity. Baxevanis *et al.,* (2004) observed that an increase in both temperature and salinity have negative influence on the overall reproductive performance and morphometric characters, and pointed out that the salinity is the prevailing environmental component and the major selective force sculpturing *Artemia* populations. Bowen *et al*., (1985) and Lenz, (1987) reported that *Artemia* inhabit the chloride, sulphate or carbonate waters and combinations of more than two anions in nature. According to Clegg and Trotman, (2002) the challenging ecological settings make *Artemia* a useful model organism for studies on evolutionary and ecological aspects of the stress response, at all levels of biological organization.

During stress, molecular chaperones interact with other proteins to modulate folding, cell localization and functionality, and to protect against irreversible denaturation. The small heat shock proteins (sHsps) are noted for their protective capability, representing the first line of defense against physiological and environmental stresses (Qui and MacRae, 2008). According to Tanguay *et al.,* (2004), in the absence of stress, all sHsp26 exists in the cytoplasm, presumably moving freely diffusing and not associated with the cytoplasmic elements, and moves in to nuclei, mitochondria, endoplasmic reticulum etc., upon stress. Further, these translocations are apparently initiated by a stress-induced acidification within the cells of the embryo. The trans-located sHsp26 is believed to function as a molecular chaperone for proteins while the embryo is under stress and during recovery (Willsie and Clegg, 2002). ArHsp22 is the third sHSP identified in diapause-destined embryos of *A. franciscana*, the others being ArHsp21 and p26 (Qiu and MacRae, (2008), Sun and MacRae, (2005), Sun *et al*., (2006). Clegg, (2007), Villeneuve *et al*., (2006)). ArHsp22 possesses an α-crystallin domain flanked by variable amino- and carboxy-terminals, all with characteristic sequence properties (Sun and MacRae, 2005).

According to Qiu and MacRae, (2008), ArHsp22 inhibits citrate synthase aggregation *in vitro* under elevated temperature, and check dithiothreitol-induced denaturation of insulin at 25^0C. Difference between the in vitro chaperone activities of ArHsp22, ArHsp21 and sHsp26 are less, and their action in the commonly used assays depends on its concentration. Exhibition of chaperone activity *in vitro* indicates that ArHsp22, ArHsp21 and p26 contribute to diapause maintenance by enhancing stress tolerance (Qiu and MacRae, 2008).

While most animals lack synthetic pathways for dispensable amino acids (DAA) necessitating its acquisition by animals from their environment, few representative species of the crustaceans, mollusca and nematodes have the ability to synthesize one or more of the vertebrate DAA (Fitzgerald and Szmant, 1997). *Artemia* have different adaptation mechanisms to overcome the extreme environmental conditions mainly through the biosynthesis of osmotically active compounds, cryoprotectants / thermoprotectants, polyols, amino acids, quaternary ammonium salts and disaccharides (Yancey *et al*., 1982).

Different studies of Clegg *et al.*, (1962, 1965, 1974, 1986, 2002, 2004) have revealed that trehalose, which constitute 15–17% of dry *Artemia* eggs is involved in the maintenance of the high ultra-structural integrity, and it was proved to protect the membrane structure and work against desiccation damage. Weisburd, (1988) and Elbein *et al.*, (2003) have established that trehalose possess the most important protective role in stabilizing and protecting membranes, proteins and allowing the anhydrobiotic organisms to survive the repeated dehydration-rehydration impacts.

Glycerol, which constitutes 2–5% of dry egg mass was hypothesized to protect embryos in stress environments (Clegg and Campagna, 2006). Wang *et al.*, (2010) reported that some biochemical components synthesized in the *Artemia* eggs also play protective roles. Hence an attempt to evaluate the effect of salinity stress on the expression of sHSP and biochemical constituents such as trehalose, carotenoids, fatty acids, amino acids, and protein was made in this study. Fatty acids are ubiquitous in all living organisms and possess an important role in energy metabolism, control the physical properties of cell membranes and act as regulators of metabolism in cells (Clark *et al.*, 1994). However, animals don’t have the ability for the de novo biosynthesis of poly unsaturated fatty acids except in the case of a few insect species under exceptional physiochemical conditions (Navarro *et al.*, 1991).

The small heat shock proteins (sHSPs) forms a distinctive molecular chaperone with well defined role in conferring tolerance to the cells upon stress (ZhijunQiu *et al.*, 2004). Plants are also known to exhibit similar phenomenon where the salt stress induces quantitative and qualitative changes in protein content of the plant cells (Wimmer, 2003).

The *Artemia* Hsp22 chaperones prevents irreversible, stress induced protein denaturation, and act as the first line of defense against salt stress. Maimbo *et al.*, (2007), Candido, (2002) and Guan *et al.,* (2004) are of the view that heat shock can induce its production in adults. Zhijun *et al.*, (2008) have reported that the mechanism by which diapausing organisms achieving reversible dormancy and increased stress tolerance is mainly by the enhanced expression of sHSP, along with the different other biochemical and morphological adaptations. Hence a variation in expression of ArHsp 22 gene in *Artemia* is subjected to salt stress is an interesting area of study. This require quantitative expression analysis using real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in “real time”. Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle and the real-time PCR data can be evaluated without gel electrophoresis, resulting in reduced experiment time and increased throughput. Finally, because reactions are run and data are evaluated in a closed-tube system, opportunities for contamination are reduced and the need for post amplification manipulation is eliminated (BioRad, USA). Thus, the present study focuses on the quantitative gene expression of ArHsp 22 and its variations in *Artemia* following exposure to salt stress.

The present study was taken up with objectives of evaluating the effect of salinity stress on survivability, biochemical constituents such as soluble protein, amino acid, trehalose and fatty acid, and the expression analysis of small heat shock gene ArHsp22 in allochthonous *Artemia* collected from India.

6.3. Materials and methods

6.3.1. Experimental animals

Artemia cysts were collected from the hypersaline waters of Kelambakam, Tamil Nadu (12^{0}47’ N 80^0 13’ E) during June 2010. The samples were brought to the wet laboratory of Central Marine Fisheries Research Institute (CMFRI), India, suitably cleaned, processed by bipartial floatation technique using brine and fresh water as detailed earlier (Sorgeloos *et al.*, 1983), and stored under refrigeration until further use. Decapsulation and hatching o*f Artemia* cysts (strain designation CKF) at the rate of 1gm L^{-1} was performed following standard procedure with suitable modifications (Sorgeloos, 1986). Freshly hatched nauplii were harvested and stocked in culture tanks (1 ton fiber reinforced plastic tanks) and cultured under standard conditions (salinity: 35‰, temperature: 23±1^{0}C). Ten percent of the culture medium was exchanged daily with fresh sea water and was fed *ad libitum* with *Isochrysis galabana* taken from the microalgae culture facility of Central marine fisheries research institute. Culture was maintained for fifteen days to be used in the experiment.

6.3.2. Experimental setup

6.3.2.1. Salinity stress treatment

Experiment was designed to study the biochemical and genetic variations in *Artemia* subjected to salt stress. They were exposed to four different salinities such as 35 (control), 100 (Treatment I), 150 (Treatment II), and 200 ppt (Treatment III) for varying durations (at 0, 6 and 24 hour). Saline solutions of 35, 100, 150 and 200 ppt were prepared by diluting the 6Msodium chloride (Sigma -Aldrich®) solution in distilled water. Each salinity condition was kept as separate treatments as outlined below.

Control: *Artemia* maintained at 35 ppt was used as the control in the experiment. In brief, adult *Artemia* individuals (1000 males and 1000 females) were randomly harvested and stocked in 25Lr in acrylic glass tanks (1 individual per 10 mL) containing 35ppt seawater, in triplicates.

Treatment I, II and III: Salinity stress experiment was carried out in 100, 150 and 200 ppt conditions and designated as Treatment I, II and III respectively. As in the control, adult *Artemia* individuals (1000 males and 1000 females) were randomly harvested and stocked in 25Lr acrylic glass tanks (1 individual per 10 mL) containing 100, 150 and 200 ppt seawater in treatment tanks I, II and III respectively, in triplicates. Prior to the experiment, *Artemia* were acclimatized to experimental conditions by progressively increasing the salinity to desired levels in all treatments. All the treatments were provided with optimum aeration, light (1500 Lux), temperature (23 ± 1^0 C) and *ad libitum* feed (*Isochrysis galabana* (45X 10^6 cells mL^{-1})).

6.3.3. Evaluation of survival rate

Survival rate of the adult *Artemia* in control and different treatments (I to III) were checked after 6 and 24hours of incubation. The survival percentages were calculated for each treatment using the following formula. Survival percentage = [Number of live animals/ (Number of live animals + Number of dead animals)] X 100.

6.3.4. Sampling for biochemical and molecular analysis:

Artemia samples were harvested from all treatment tanks and control after 6 and 24 hours of incubation. Samples required for the biochemical estimations (40gm) were, lyophilized (Martin Christ Alpha-1-4 LD freeze-drier, Osterode, Germany) and stored at -80^0 until analysis. Samples required for gene expression study (30 individuals) were preserved in individual Eppendorf Safe-Lock Tubes 1.5 mL with RNAlater solution (1.3ml) (Sigma Aldrich, USA).

6.3.5. Biochemical analysis

6.3.5.1. Estimation of soluble protein

Soluble protein content were estimated from the lyophilized *Artemia* samples (100mg) using established method in triplicate (Lowry *et al*. 1951). Bovine serum albumin (BSA, 4 mg/mL) in distilled water was used as standard for the estimation. The absorbance of the protein aliquot was measured at 660 nm in an ultraviolet/visible spectrophotometer (Cary 50, Varian, USA) against the reagent blank. The protein percentage of each sample was calculated from the standard curve of BSA.

6.3.5.2. Estimation of total carotenoids

Total carotenoid content in the *Artemia* from each treatment was determined using 100mg of lyophilized sample by the established methods in triplicates (Tolasa *et al*. 2005; Olson, 1979). In brief, the samples (100 mg) were ground with anhydrous Na_2SO_4 (500mg) and the contents were dissolved in $CHCl_3$ (1 mL). The homogenates were incubated overnight at 0^oC and clarified by centrifugation (10,000 rpm for 20 min). The clarified $CHCl_3$ layer (0.4 mL) was diluted (10 times) with ethanol and scanned using spectrophotometer (Cary 50, Varian, USA) through λ=200 to 800. The samples were then quantified by reading the absorbance at 475 nm. Concentration of carotenoid was calculated following the formula: total carotenoid (µg / gm) = (absorbance X 10)/ (0.25 X sample weight (gm)), dilution factor = 10, extinction coefficient = 0.25.

6.3.5.3. Estimation of amino acids

Amino acid content of the *Artemia* samples was estimated following the Pico-Tag method (Heinrikson and Meredith, 1984) in triplicates. *Artemia* samples (100mg) from each treatment was digested with HCl (6N, 10mL) at 110°C for 24 hours, and flash evaporated thrice using distilled water (20 mL X 3) to remove the acid. Acid-free samples were then made up to 5 mL with HCl (0.05 N), and analyzed using reversed-phase binary gradient high performance liquid chromatography (HPLC, Waters reversed-phase PICO.TAG amino acid analysis system). Samples (PTC amino acid derivatives) were injected in triplicate, and the output was analyzed using BREEZE software. The quantification of amino acids was carried out by comparing the value of the sample with the standard and the results were expressed as the mean of triplicates.

6.3.5.4. Estimation of trehalose

Trehalose contents of the *Artemia* under various treatments were estimated following the established methods with suitable modifications (Carpenter and Hand, 1986; Jermyn, 1975). *Artemia* samples (50 mg) were homogenized in 6% perchloric acid (2ml) and the supernatant neutralized by saturated sodium carbonate and the perchloric salts were removed. The homogenates were made up to10 ml with distilled water and from each homogenate 0.5 and 1.0 ml samples were taken along with the working standards (0.2, 0.4, 0.6 and 0.8 ml of glucose) and were made up to 1 ml with ice cold distilled water. To this, 4 ml of Anthrone reagent was added and digested for 8 minutes at 100°C in a water bath and values were estimated at 630 nm in a spectrophotometer (Cary 50, Varian, USA). Trehalose content in the sample was calculated from the graph using the following equation. Amount of Trehalose in the Sample = [(Sample value from the graph (mg)/ (Volume of sample)) X (Total Volume of extract (ml) / (Weight of sample in (mg))] X 100.

6.3.5.5. Estimation of fatty acids

Total lipid was extracted with $CHCl_3$/MeOH (60 mL, 2:1, v/v) and water (20 mL) in triplicates (Bligh and Dyer 1959). The $CHCl_3$ layer was processed to recover the triglycerides and saponified with alkaline reagent (3 mL, 0.5 N KOH/MeOH). The saponified materials were reacted with the methylating mixture (14% BF_3/CH_3OH) to yield methyl esters (FAME) and subsequently extracted with *n*-hexane/H_2O (1:2, v/v) mixture (Metcalfe *et al*. 1966). Aqous layer was removed and the *n*-hexane layer was recovered and concentrated in vacuum, reconstituted in petroleum ether and stored at -20°C. Fatty acids composition was analyzed using a Perkin Elmer Auto System XL, Gas chromatograph (Perkin Elmer, USA) equipped with a flame ionization detector.

6.3.6. Molecular analysis

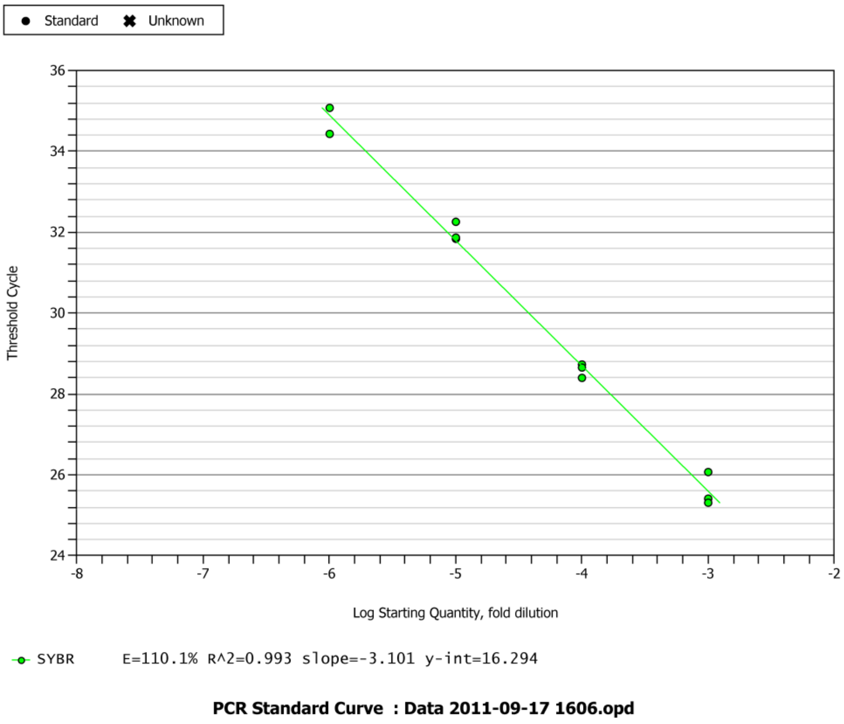
6.3.6.1. RNA isolation and cDNA synthesis

Total RNA from individual *Artemia* were extracted using the “GenElute^{TM}Mammalian Total RNA Miniprep kit” (Sigma Aldrich, USA), following the manufacturer's instructions, and stored at−80 °C. DNAse I treatment was done to remove genomic DNA contamination from the samples using “DNase I- amplification grade” (Invitrogen; United Kingdom), following the manufacturer's instructions. First-strand cDNA was synthesized using the “iScriptc DNA synthesis kit” (BioRad, USA). Each 20 µL reaction mixture contained total RNA 300 ng, 5x I Script reaction mix 4.0 µL, iScript reverse transcriptase 1.0 µL and nuclease free water. Synthesis of cDNA was performed in a PCR Thermalcycler cycler (BioRad, USA) according to the following the temperature ramp. Initial hybridization step of 5 min at 25°C, followed by reverse transcription (RT) for 45 min at 42 °C and a final cycle of 5 min at 75^0C was carried out. The synthesized cDNA samples were kept at−80 °C. The samples incubated without the addition of reverse transcriptase were used as controls.

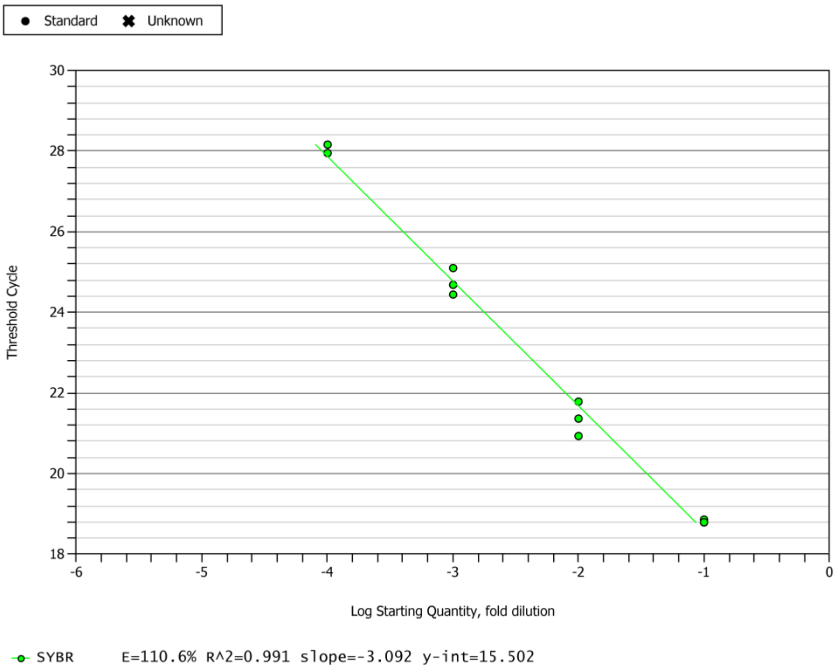
6.3.6.2. Real time–PCR and quantitative analysis

Real time–PCR using cDNA was carried out to study the expression of ArHsp22 gene as it allows detection and quantitative measurement and analysis of the amplified product being accumulated as the reaction progresses, in “real time”. Housekeeping genes viz.β actin and GADPH (Chandna *et al.,* 2012) were also amplified along with ArHsp22 gene, using the gene specific primers, as endogenous control to be used for normalization of amplification data. Real time PCRs were performed using an IQ 5 thermal cycler sequence detection system (BioRad, USA). The PCR thermal cycling conditions for all these genes were as follows: an initial denaturation step at 95 °C for 5 min, followed by 40 cycles consisting of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec and elongation at 72^0C for 45 sec. The reaction mixture (25 μL) contains 10.5 μL of sterile Milli-Q water, 0.75 μL of each primer (10 μM), 1.5 μL of template cDNA and 12.5 μLiQ^{TM} SYBR green supermix (BioRad, USA). Non template controls (NTC) without cDNAwere also subjected to the same procedure to exclude or detect any possible contamination or carryover. The results were normalized using the β actin and GADPH cDNA amplifications run on the same plate. In a real time RT–PCR analysis, target DNA quantification is mainly based on the Threshold Cycle (CT). The ct value indicates the first amplification cycle at which the fluorescence signal is greater than the minimal detection level, indicating that target products become detectable and can be subjected to the relative quantization study (Gizinger, 2002). The PCR efficiency of the three genes was measured by performing a 10 fold serial dilution of positive control template to generate a standard curve, and by plotting the CT as a function of log10 of the template. The slope in the standard curve for the amplification of the β actin, GAPDH and ArHsp22 was -3.085, -3.092 and -3.101 respectively. The efficiency of the β actin, GAPDH and ArHsp22 genes were found to be 110.9, 110.6 and 110.1% respectively (Fig. 6.1). PCR efficiency values ranging from 95 and 110%, with ≤0.1 slope value was considered as the optimum. Threshold cycle (CT) values were transformed to log2 (due to the doubling function of PCR) to generate the relative expression levels.

A

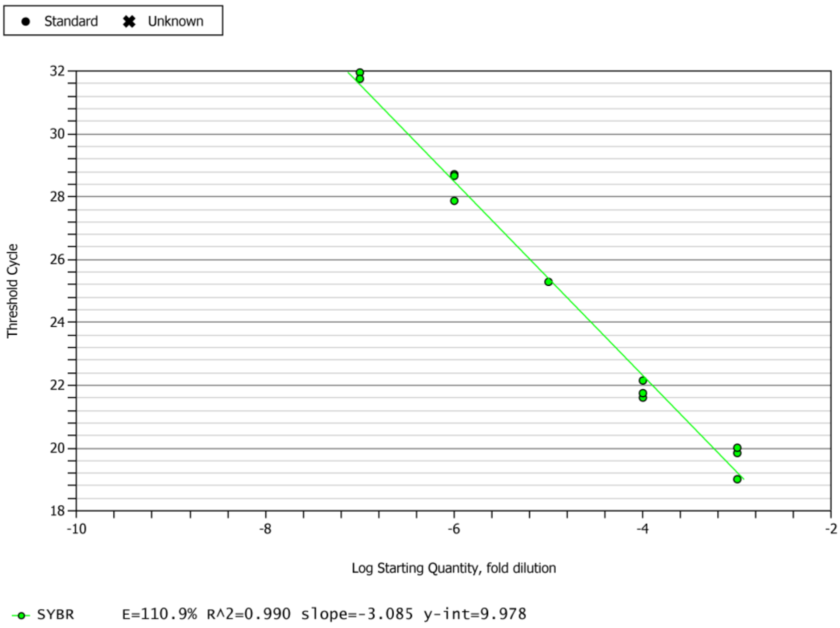


B



PCR Standard Curve : GADPH Data 2011-09-19 1118.opd

C



PCR Standard Curve : Beta actin Final.opd

Fig.6.1 PCR efficiency of β actin, GAPDH and ArHsp22 genes

6.3.7. Statistical analyses

Means of all parameters were tested for the significance of difference by analysis of variance (ANOVA) using the Duncan multiple tests by SPSS programme 13.0 (SPSS Inc, Chicago, USA) ($P<0.05$).

6.4. Results

6.4.1. Mortality percentage of *Artemia* subjected to salt stress

In control (35ppt), no moralities were observed up to 24 hours of incubation. In the case of treatments I (100 ppt) and II (150 ppt), mortalities started appearing after the first 6 hours of incubation and continued up to 24 hours to reach 2.84±1.05 and 2.89±1.44 respectively. Treatment III (200 ppt) showed the highest mortality. After incubation the mortality was 2.74 % which increased sharply to reach 23.06 % by 24th hour of incubation ($P<0.05$) (Fig. 6.2).

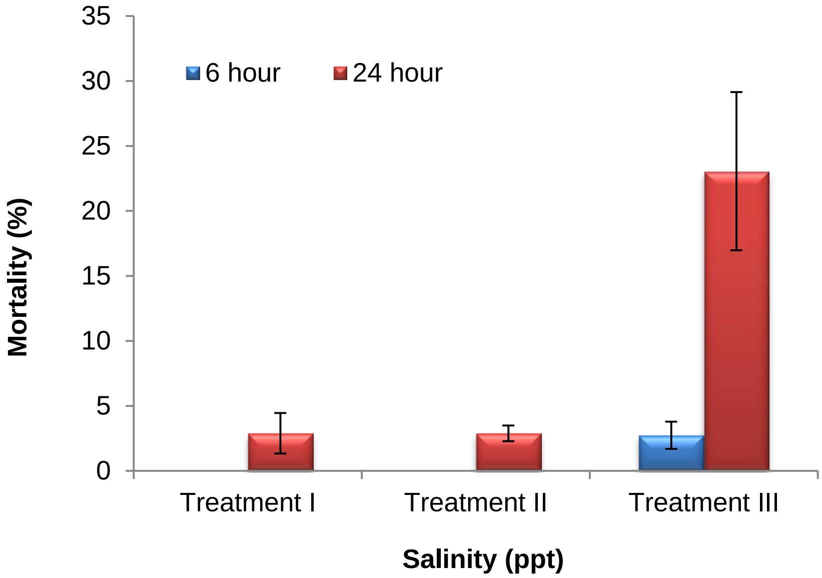


Fig.6.2. Mortality percentage of *Artemia* subjected to salt stress

6.4.2.Variation in soluble protein content in *Artemia* under salt stress

Soluble protein content in the *Artemia* which were subjected to salinity stress varied significantly when compared to the control (Table 6.1). At 6 hours post incubation, except in treatment I (24.41 ± 0.1%), a significant reduction in the soluble protein levels was observed in all the treatments (16.75 ± 0.01 and 18.67 ± 0.01%). At 24 hours of incubation, the soluble protein level showed a significant increase in treatment II (23.24 ± 0.01%), while a significant reduction was observed in treatment III (15.24 ± 0.08) ($P<0.05$). During this period, the soluble protein level in treatment I exhibited a marginal reduction (22.88 ± 0.02) when compared to the previous observation at 6 hours post incubation. The highest soluble protein level (24.41 ± 0.01%) was observed in treatment I at 6 hours incubation while the lowest value was observed for treatment II at 24 hours post-incubation (15.24 ± 0.08%).

Table 6.1 Trehalose, Protein and Carotenoid content of *Artemia* adults under different salinity conditions

Treatment/ time	Protein (%)	Carotenoid (µg/gm)	Trehalose (%)
C 0h	22.56 ± 0.04^{a}	154.84 ± 1.11^{a}	2.50 ± 0.08^{a}
C 6h	21.89 ± 0.03^{a}	158.71 ± 1.08^{a}	2.49 ± 0.08^{a}
C 24h	22.02 ± 0.04^{a}	159.65 ± 1.06^{ac}	2.56 ± 0.08^{ab}
TI 6h	24.41 ± 0.1^{a}	126.95 ± 1.50^{b}	4.66 ± 0.35^{b}
TI 24h	22.88 ± 0.02^{a}	199.76 ± 1.09^{c}	4.29 ± 0.77^{cf}
TII 6h	16.75 ± 0.01^{bcd}	151.45 ± 1.03^{d}	3.60 ± 1.18^{d}
TII 24h	23.24 ± 0.01^{a}	200.34 ± 0.50^{e}	4.74 ± 0.92^{e}
TIII 6h	18.67 ± 0.01^{cb}	103.76 ± 1.09^{f}	4.24 ± 0.34^{f}
TIII 24h	15.24 ± 0.08^{dc}	110.71 ± 1.08^{g}	2.61 ± 0.07^{a}

6.4.3. Changes in total carotenoid content in *Artemia* under salt stress

At 6 hours post incubation, the total carotenoid content showed a significant reduction in treatments I, II and III (126.95±1.5 µg/gm, 151.45±1.03 µg/gm and 103.76±1.09 µg/gm respectively) when compared to the control (158.71±1.08 µg/gm). However, at the 24th hour it showed a significant increase in treatments I and II (199.76±1.09 µg/gm and 200.34±0.50 µg/gm respectively) compared to the values at 6th hour as well as the control (159.65±1.06 µg/gm). While in treatment III, the increase at 24th hour was only marginal (110.71±1.08 µg/gm) compared to the 6th hour value, and it failed to reach the normal control values (159.65±1.06 µg/gm) ($P<0.05$). The highest value for total carotenoid was observed in treatment II at 24 hours post-incubation while the lowest value (103.76±1.09 µg/gm) was observed at 6 hours post-incubation in treatment III (Table 6.I).

6.4.4. Variation in trehalose content in *Artemia* under salt stress

Trehalose content in all the three treatments showed an increase at both 6th and 24th hour of exposure compared to the control (Table 6.I), with the highest level being at 6th hour in treatment II. The increased trehalose level at 6th hour of incubation in treatments I, II and III were 4.66 ± 0.35 %, 3.60 ± 1.18 % and4.24 ± 0.34 % respectively, and were significantly higher than the control (2.49 ± 0.08 %). At 24th hour of incubation, though the values showed a significant reduction when compared to the values at 6th hour (4.29 ± 0.77 %, 4.74 ± 0.92 % and 2.61 ± 0.07 % for I,II and III treatments respectively), they were still higher than the control (2.56 ± 0.08 %) ($P<0.05$).

6.4.5. Variation in fatty acid content in *Artemia* under salt stress

The level of total saturated fatty acids showed a reducing trend following exposure to salt stress. After 6 hours of incubation, while the reduction was only marginal in treatments I and III (42.57 % and 42% respectively), the reduction was significant (35.84%) in treatment II, compared to the control (44.6%). At 24th hour, the levels were further reduced and reached 39.35%, 33.83% and 38.27% in treatments I, II and III respectively. The lowest total saturated fatty acid was observed in treatment II at 24th hour of incubation (33.83%). The increase in salinity was found to be leading to a progressive reduction in the saturated fatty acids level. Among the 10 different saturated fatty acids analyzed, the 16:00 was observed to be contributing the major share, followed by 18:00 (Table 6.2).

Table 6.2. Fatty acid content of *Artemia* adults under different salinity conditions

Fatty acid		Control		Treatment I		Treatment II		Treatment III	
	0h	6h	24h	6h	24h	6h	24h	6h	24h
12:00	0.14	1.17	3.90	0.55	0.93	1.95	1.15	2.29	0.55
13:00	0.14	0.32	0.30	0.21	0.17	0.65	0.04	0.18	0.27
14:00	3.87	3.37	4.39	4.71	6.10	6.52	5.24	3.55	4.45
15:00	3.32	7.19	0.90	5.62	0.41	0.28	0.15	0.40	1.03
16:00	22.84	20.59	20.89	18.03	18.98	16.66	15.00	22.17	19.10
17:00	1.21	0.97	3.40	3.19	2.55	0.74	2.30	1.21	3.49
18:00	8.41	8.87	8.60	7.21	8.30	6.89	6.81	9.35	8.15
20:00	1.38	0.78	1.40	0.90	0.87	0.84	0.80	1.12	0.75
22:00	1.03	1.04	0.80	1.80	0.87	0.84	2.07	0.54	0.27
24:00	0.74	0.32	0.20	0.35	0.17	0.47	0.27	1.08	0.21
∑ SFA	43.08	44.62	44.78	42.57	39.35	35.84	33.83	42.00	38.27
14:1n7	3.94	1.10	5.80	0.49	4.53	7.26	4.02	5.76	5.75
16:1n7	4.99	4.14	3.40	4.02	4.47	3.35	3.52	2.92	3.15
18:1n9	18.98	19.95	15.99	14.63	26.12	12.38	14.92	18.89	16.02
17:01	0.50	1.10	1.90	1.18	0.35	1.68	1.19	0.54	0.68
20:1n11	0.50	0.39	0.70	1.04	0.87	1.86	0.99	1.26	2.40
∑ MUFA	28.91	26.68	27.79	21.36	36.34	26.53	24.64	29.00	28.00
18:2n6	4.43	4.34	4.60	4.16	4.24	3.26	4.13	2.56	3.63
18:3n6	3.83	4.08	3.10	4.30	3.89	2.89	4.06	2.11	3.08
18:3n3	0.89	2.07	1.50	1.59	1.22	2.79	1.07	1.80	1.71
20: 2n6	3.90	3.89	1.90	5.48	4.93	2.98	6.16	3.37	4.11
20:3n6	0.92	0.52	0.60	0.97	0.35	1.21	0.92	3.37	1.10
20:4n6	0.67	1.36	1.00	1.53	0.29	1.30	0.96	0.94	1.03
20:5n3	3.55	3.11	3.90	4.58	2.03	4.00	3.63	0.67	4.31
22:5n3	3.02	0.91	2.70	1.66	0.29	2.14	1.19	0.45	0.96
22:6n3	3.10	3.04	4.70	7.63	3.72	12.29	10.14	6.03	6.30
∑ PUFA	24.31	23.32	24.00	31.90	20.96	32.86	32.26	21.00	26.23
∑ n3	10.56	9.13	12.80	15.46	7.26	21.22	16.03	8.95	13.28
∑ n6	13.75	14.19	11.20	16.44	13.70	11.64	16.23	12.35	12.95
∑ C18PUFA	9.15	10.49	9.20	10.05	9.35	8.94	9.26	6.47	8.42
∑ C20PUFA	9.04	8.88	7.40	12.56	7.60	9.49	11.67	8.35	10.55
n3/n6	0.77	0.64	1.14	0.94	0.53	1.82	0.99	0.72	1.03
∑PUFA/∑SFA	0.56	0.52	0.54	0.75	0.53	0.92	0.95	0.50	0.69
EPA/AA	4.51	2.29	3.90	2.99	7.00	3.08	3.78	0.71	4.18
∑DHA/∑EPA	0.87	0.98	1.21	1.67	1.83	3.07	2.79	9.00	1.46

ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; ΣPUFA: total polyunsaturated fatty acids; ∑ n-3: total n3 polyunsaturated fatty acids; ∑ n-6: total n6 polyunsaturated fatty acids; ∑ C18PUFA: total C18 polyunsaturated fatty acids; ∑ C20PUFA: total C20 polyunsaturated fatty acids; EPA: Eicosapentanoic acid; AA: Arachidonic acid; total n3 polyunsaturated fatty acids. Data presented as the mean values of three replicates.

Monounsaturated fatty acid (MUFAs) values following 6 hours of incubation varied in all the three different treatments with treatment I showing a significant reduction (21.36%), treatment II remaining unchanged (26.53%) and treatment III showing a significant increase (29.00%) when compared to the control (26.68%). After 24 hours, total MUFA content decreased in treatment II and III to 24.64% and 28% respectively, whereas it increased substantially in treatment I (36.34%), when compared to the values at 6 hours. However, the MUFA content at 24th hour was higher than the control, except in treatment II. Among all the treatments, the highest MUFA content was observed in treatment I at 24 hours incubation and the lowest in treatment II at 24 hours incubation. Of the 5 MUFAs analyzed 18:1n9 contributed the major share followed by 14:1n7 and 16:1n7 (Table 6.2).

The polyunsaturated fatty acids (PUFAs), after 6 hours of incubation, showed a significant increase in treatment I and II (31.9% and 32.86% respectively) while there was a significant reduction in treatment III (21%) when compared to the control (23.32%). At the 24th hour, the PUFA content showed a sharp reduction in treatment I when compared to the values at 6 hours and control (24%), while in treatment II the values remained same as that at 6 hours but was still significantly higher than the control (24%). In treatment III the PUFA content showed a marginal increase (26.23%) when compared to the values at 6 hour and control (24%). Among the different treatments, the highest PUFA was observed in treatment II at 6 and 24 hours post-incubation (Table 6.2) while the lowest value was recorded in treatment I at 24 hours post-incubation.

The 20:5n3 content, increased in treatments I and II (4.58% and 4% respectively), showed a decrease in treatment III (0.67%), when compared to the control (3.11%) at 6 hours. However, by the 24th hour, the trend reversed with treatment I showing considerable reduction (2.03%), treatment II with a marginal reduction (3.63%), while treatment III exhibited a significant increase (4.31%) compared to the control (3.9%) and 6 hour incubation (0.45%). Among the different treatments, 20:5n3 content remained high and stable in treatment II at 6 and 24 hours of incubation (Table 6.2).

In case of 22: 6n3, significant increase was observed in all the three treatments at 6 hours of incubation (7.63%, 12.29% and 6.03% for I, II and III treatments respectively) when compared to the control (3.04%). At 24 hours marginal reduction was observed in treatment I and II (3.72% and 10.14% respectively) when compared to values at 6 hours and control. In treatment III the 22: 6n 3 content showed a marginal increase (6.3%) when compared to the values at 6 hours and control. Among the different treatments, 22: 6n3 content remained high and stable in treatment II at 6 and 24 hours of incubation (Table 6.2).

Highest ∑n3 value was observed in treatment II at 6 and 24 hours (21.22 and 16.03 respectively) while the highest ∑n6 value was observed in treatment I at 6 hours (16.44) and treatment II at 24 hours (16.23). The n3/n6 ratio was the highest (1.82) in treatment II at 6 hours, and was the lowest (0.53) in treatment I at 24 hours. The ∑PUFA/∑SFA values were highest in treatment II at 6 and 24 hours (0.92 and 0.95 respectively) while the lowest values were recorded for treatment III at 6 hours (0.50). Highest EPA/AA value (7.0) was recorded in treatment I at 24 hours incubation while the lowest (0.71) was observed for treatment III at 6 hour incubation. The EPA/AA value remained at the same levels in treatment II at both 6 and 24 (3.08 and 3.78 respectively) hours of incubation. The increase in ∑DHA/∑EPA ratios in different treatments varied from marginal to significant with the highest value observed in treatment III (9) at 6 hours and the lowest at 24 hours (1.46) (Table 6.2).

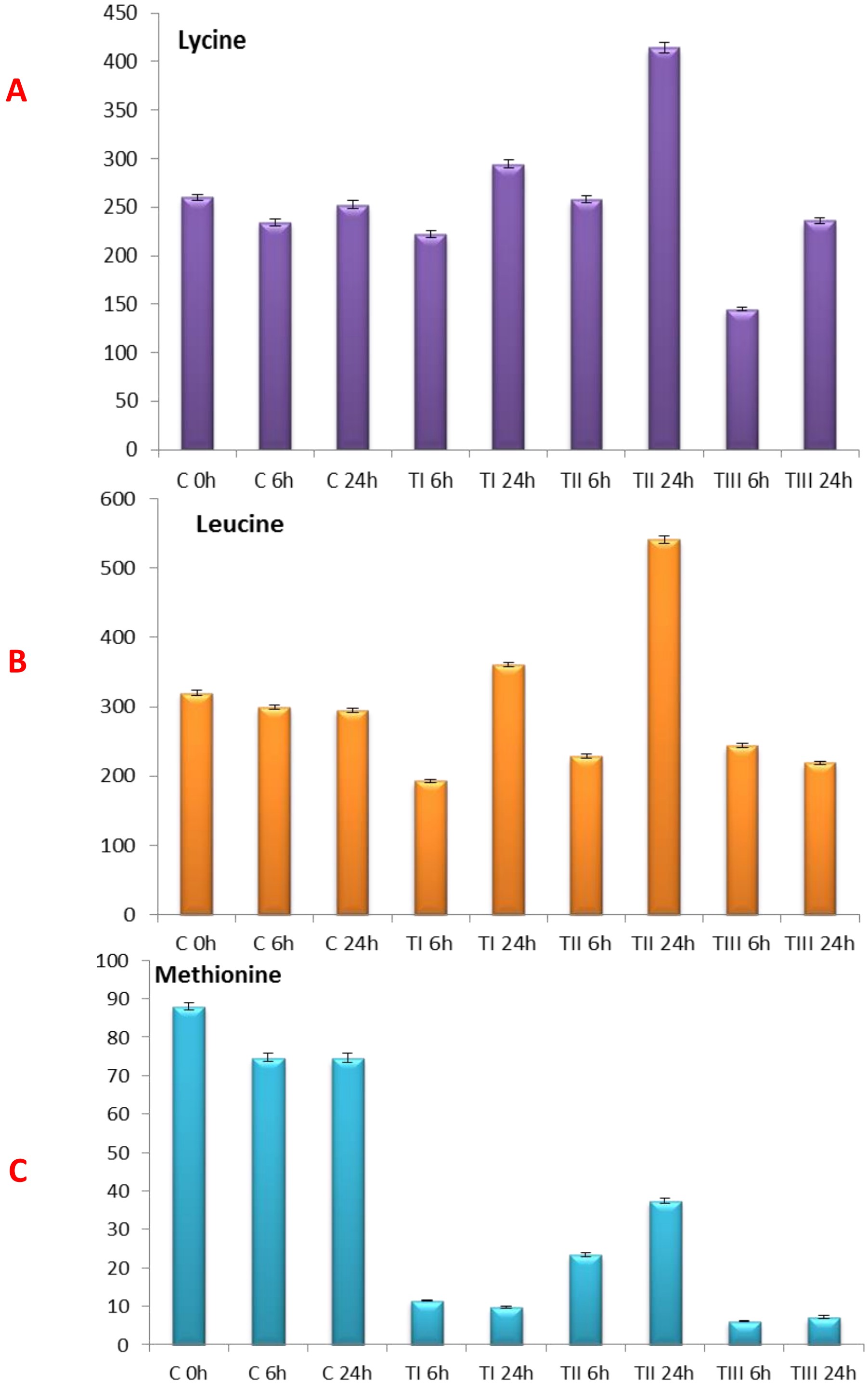
6.4.6. Variation in *amino acid* content in *Artemia* under salt stress

When exposed to high salinity regimes for 6 and 24 hours, the total amino acid content showed a steady increase (Table 6.3). At the 6th hour, the total amino acid content increased to 2784.6, 3348.32 and 3690.84 mg/100gm in treatment I, II and III respectively, compared to 2307.74 mg/100gm in control. At the 24th hour, the amino acid content was significantly high in treatments I and II (5357.93 and 8022.79 mg/100gm) when compared to the control (2554.3 mg/100gm) while in treatment III a marginal reduction (2980.44 mg/100gm) was observed compared to the value at 6 hours, but was still higher than the control (2554.3 mg/100gm). The same pattern was observed for DAA and IAA values in *Artemia* subjected to salinity stress (Table 6.3) (Fig.6.3). Among all the treatments, highest amino acid content was observed in treatment III after 24 hours of incubation (8022.79 mg/100gm).

The "branched chain amino acids" (BCAA) such as Isoleucine, leucine and valine slightly increased at 6 hours post-incubation in different treatments and continued up to 24 hours except in treatment III where a slight reduction was observed (Table 6.3).

Table 6.3. Amino acid content of *Artemia* adults under different salinity conditions

Treatment (I to IV)	Control 0h	Control 6h	Control 24H	T I 6h	T I 24h	T II 6h	T II 24h	T III 6h	T III 24h
Alanine	109.89 ± 10.0^{a}	101.17 ± 9.00^{a}	106.13 ± 7.58^{a}	112.69 ± 7.50^{a}	231.23 ± 15.1^{d}	133.69 ± 8.50^{b}	314.67 ± 15.0^{e}	153.68 ± 7.77^{c}	138.28 ± 12.5^{bc}
Arginine	162.91 ± 11.1^{a}	270.83 ± 10.1^{b}	456.09 ± 15.1^{c}	399.55 ± 10.0^{b}	824.54 ± 15.0^{d}	467.34 ± 12.0^{c}	1227.7 ± 25.4^{e}	607.17 ± 25.2^{f}	423.88 ± 15.1^{b}
Aspartine	195.95 ± 15.0^{a}	244.83 ± 10.0^{b}	199.97 ± 10.0^{a}	259.86 ± 10.0^{b}	531.35 ± 15.1^{c}	410.14 ± 20.0^{d}	780.26 ± 20.0^{e}	346.45 ± 10.3^{f}	298.17 ± 15.9^{g}
Cystine	372.04 ± 12.5^{a}	120.12 ± 10.0^{a}	62.073 ± 2.00^{c}	150.53 ± 10.0^{d}	196.63 ± 6.50^{e}	82.763 ± 5.01^{f}	329.09 ± 15.0^{g}	112.69 ± 7.50^{a}	75.443 ± 1.26^{c}
Glutamine	294.52 ± 15.0^{a}	330.82 ± 15.0^{b}	323.85 ± 10.1^{b}	373.71 ± 12.6^{c}	751.74 ± 10.4^{d}	529.24 ± 17.7^{e}	1125.8 ± 25.0^{e}	501.05 ± 20.0^{f}	426.29 ± 14.8^{g}
Glycine	95.45 ± 4.50^{a}	105.10 ± 5.00^{ab}	143.03 ± 8.53^{c}	116.85 ± 11.0^{b}	270.84 ± 10.1^{d}	143.95 ± 8.00^{c}	400.05 ± 18.0^{e}	220.05 ± 10.0^{f}	143.79 ± 9.57^{c}
Proline	122.71 ± 7.50^{a}	83.166 ± 3.01^{b}	114.56 ± 9.50^{a}	95.1 ± 5.00^{b}	247.57 ± 12.0^{d}	95.5 ± 5.07^{b}	325.56 ± 14.6^{e}	194.47 ± 9.03^{f}	123.63 ± 7.75^{a}
Serine	136.11 ± 5.36^{a}	161.16 ± 10.1^{b}	180.73 ± 11.0^{bc}	192.29 ± 7.50^{c}	325.75 ± 15.0^{e}	224.29 ± 12.8^{d}	514.13 ± 15.0^{f}	232.22 ± 11.5^{d}	170.55 ± 10.0^{b}
DAA	1489.61^{ab}	1417.21^{b}	1586.46^{bc}	1700.61^{cd}	3379.67^{e}	2086.93^{f}	5017.39^{g}	2367.81^{h}	1800.05^{d}
IAA	913.20^{a}	890.53^{a}	967.84^{ab}	1083.99^{bc}	1978.26^{e}	1261.39^{cd}	3005.40^{f}	1323.04^{d}	1180.39
Total AA	2402.81^{a}	2307.74^{a}	2554.30^{b}	2784.60^{c}	5357.93^{d}	3348.32^{e}	8022.79^{f}	3690.84^{g}	2980.44^{h}
IAA/DAA	0.61	0.63	0.61	0.64	0.59	0.60	0.60	0.56	0.66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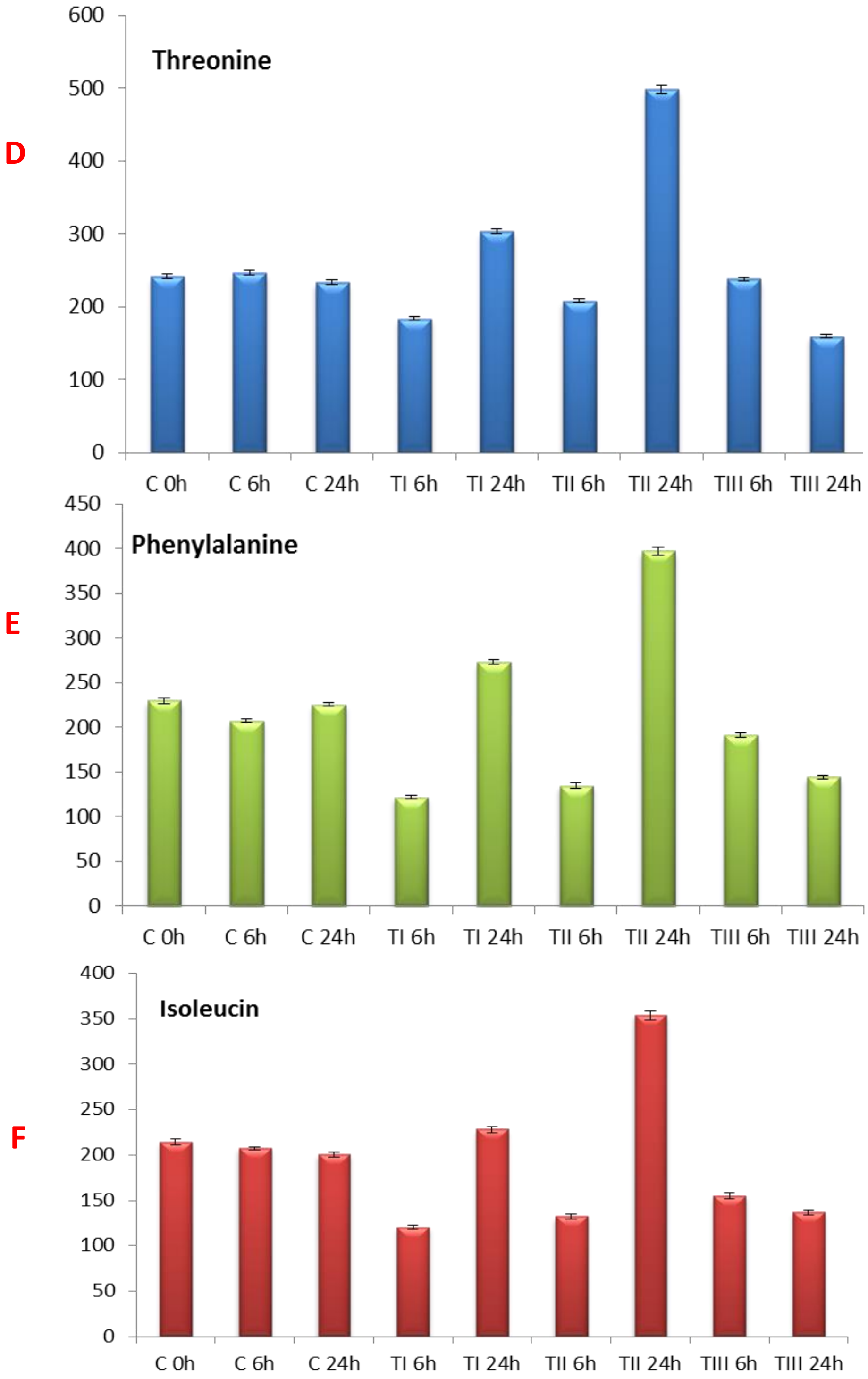
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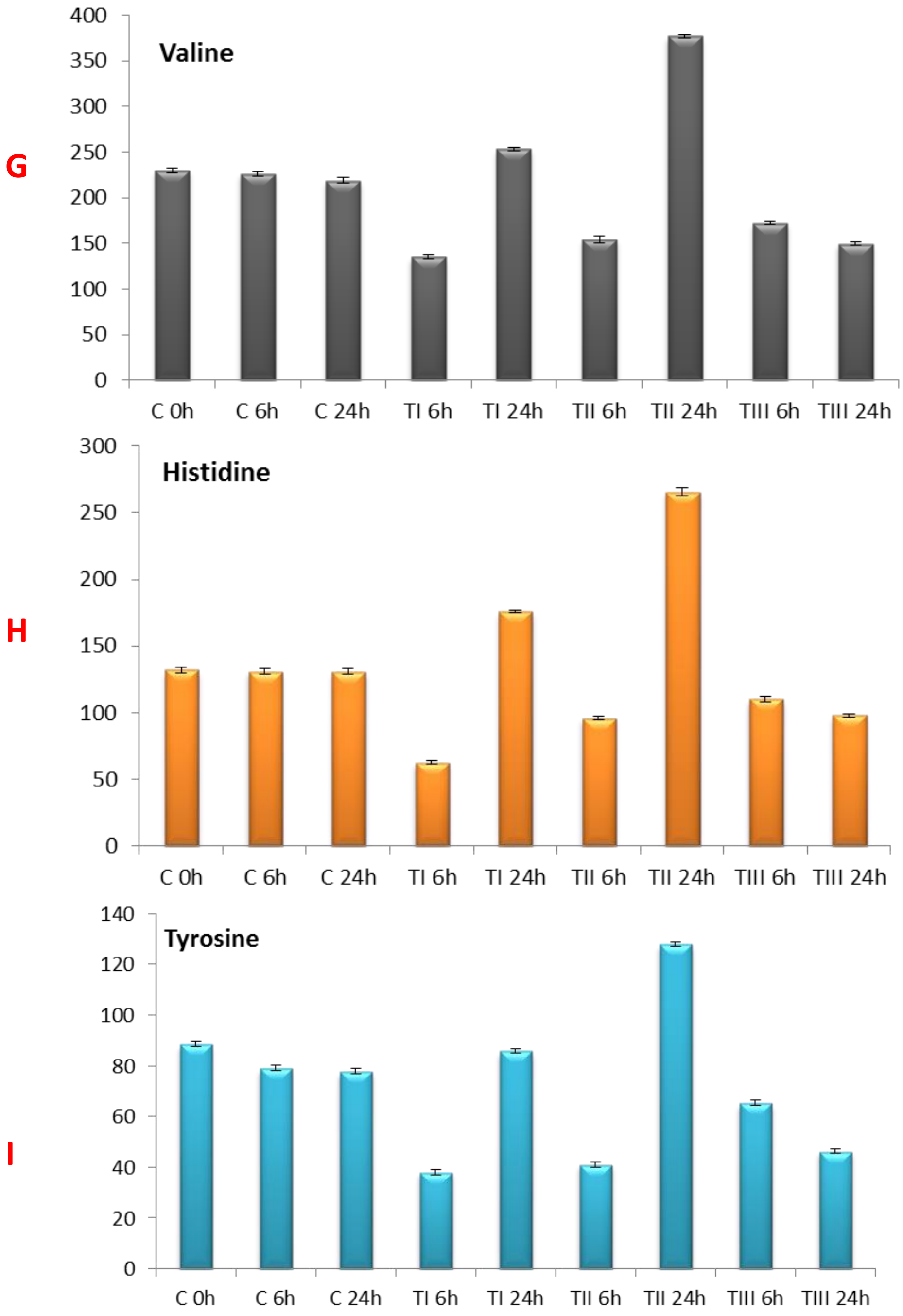


X axis: Control at 0, 6 and 24 hour and Treatments I, II, III at 6 and 24 hours
Y axis: Amino acid represented in 100gm/mg
Graph Bars represents the average amino acid (triplicate) with standard deviation

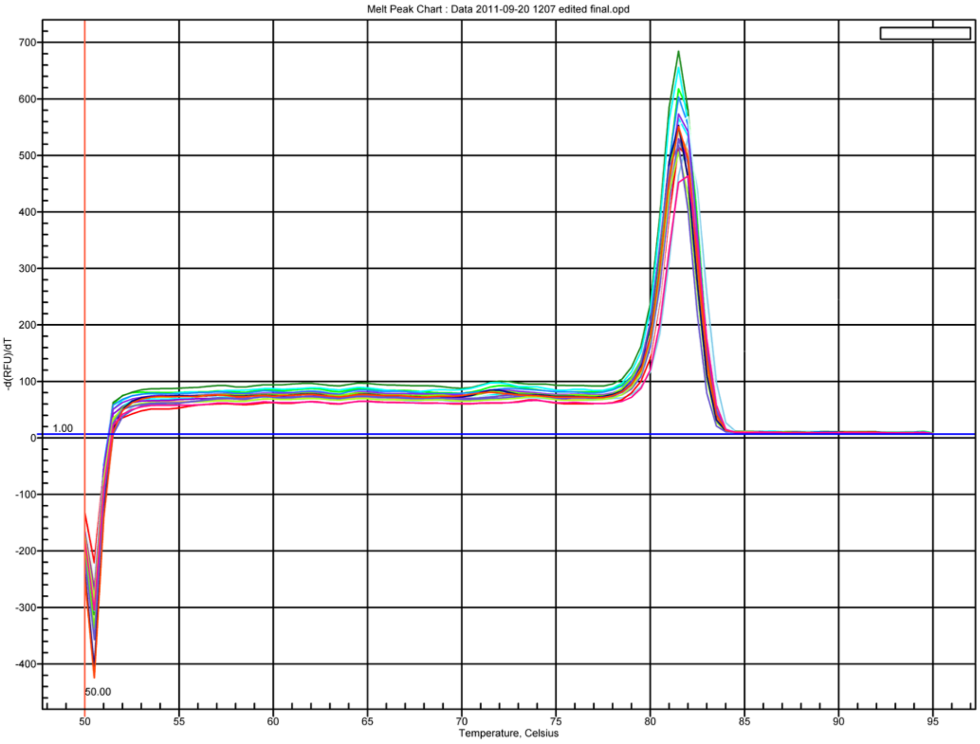
Fig.6.3 (A, B, C, D, E, F, G, H and I). IAA content of *Artemia* adults under different salinity conditions

6.4.7. ArHsp 22 gene expression in *Artemia* under salt stress

Quantitative expressional variations of the gene coding for small heat shock protein of *Artemia* ArHsp 22 showed increase following exposure to salinity stress. SYBR® Green I based real-time RT-PCR was used for quantification of the mRNA transcribed from the ArHsp 22gene, since it is a proven and accurate method quantification of RNA levels in animals (Bustin and Nolan, 2004; Lekanne Deprez *et al.*, 2002; Schmittgen *et al.*, 2000; Wilhelm and Pingoud, 2003). Two reference genes viz., β actinn and GAPDH were used as internal controls since according to Huggett *et al*., (2005) the use of reference genes as internal controls is the most common and accepted method for the normalization of cellular mRNA data.

Melt curve dissociation analysis showed specific single peaks for ArHsp22, β actin and GAPDH genes in real time RT-PCR analysis indicating the specificity of the reaction (Fig 6.4).

A



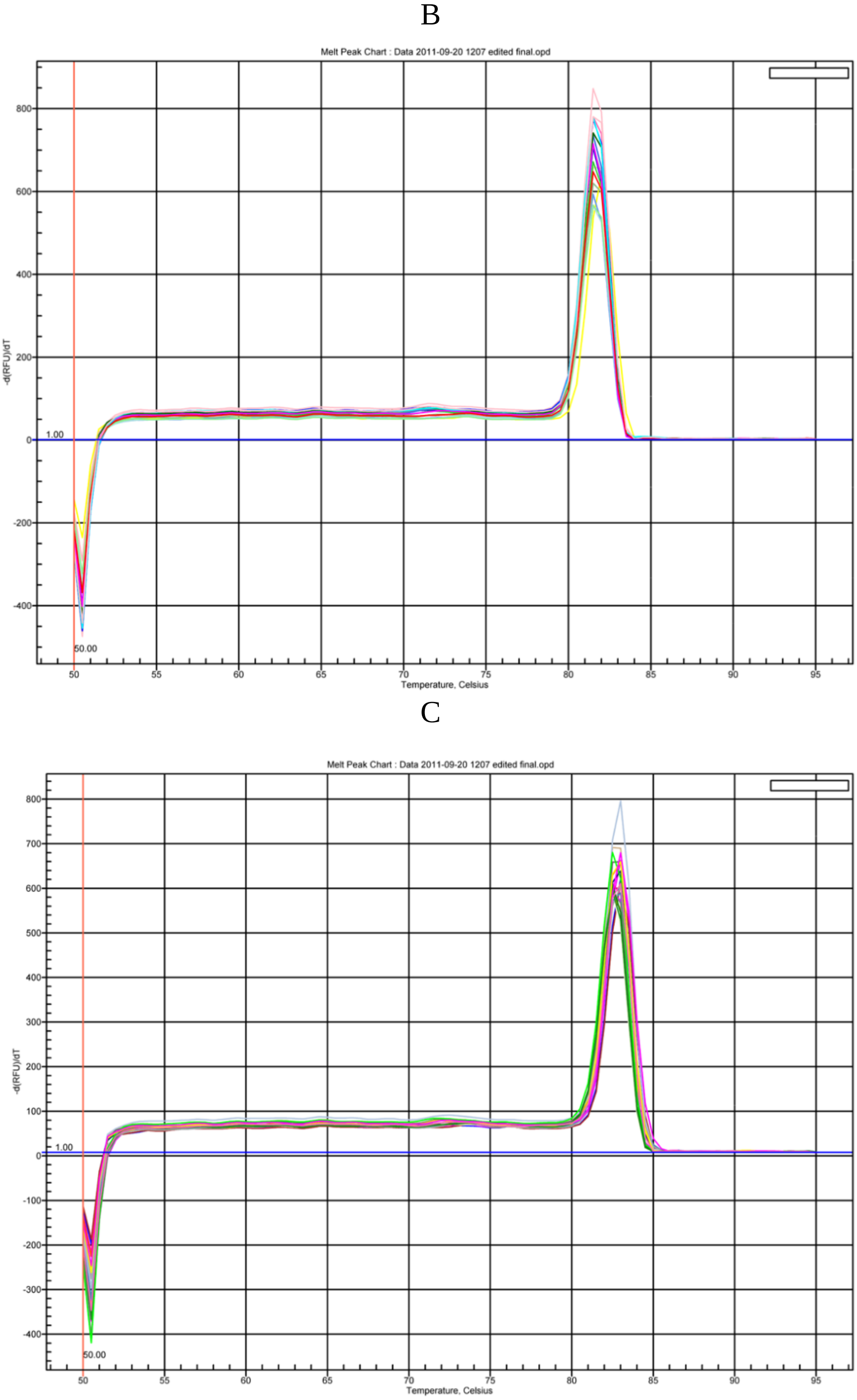


Fig.6.4. Melt curve dissociation analysis of ArHsp22 in graph A, β actin in graph B and GAPDH in graph C

Normalization of the target gene expression was carried out because this process controls variations in extraction yield, reverse-transcription yield, and efficiency of amplification etc., thus enabling comparisons of mRNA concentrations across different samples (Bustin *et al*., 2009). In treatments II and III the expression of ArHsp 22 gene showed a sharp rise at 6 hours of incubation, with the maximum expression being in treatment II. On the contrary, the increase in treatment I at 6 hours of incubation was only marginal. However, at 24 hours post-incubation, the ArHsp22 gene expression was considerably reduced in all treatments when compared to the 6th hour level (Fig. 6.5). Irrespective of the incubation period, the normalized expression of ArHsp22 gene was highest in treatment II (150 ppt) at 6 hours of incubation.

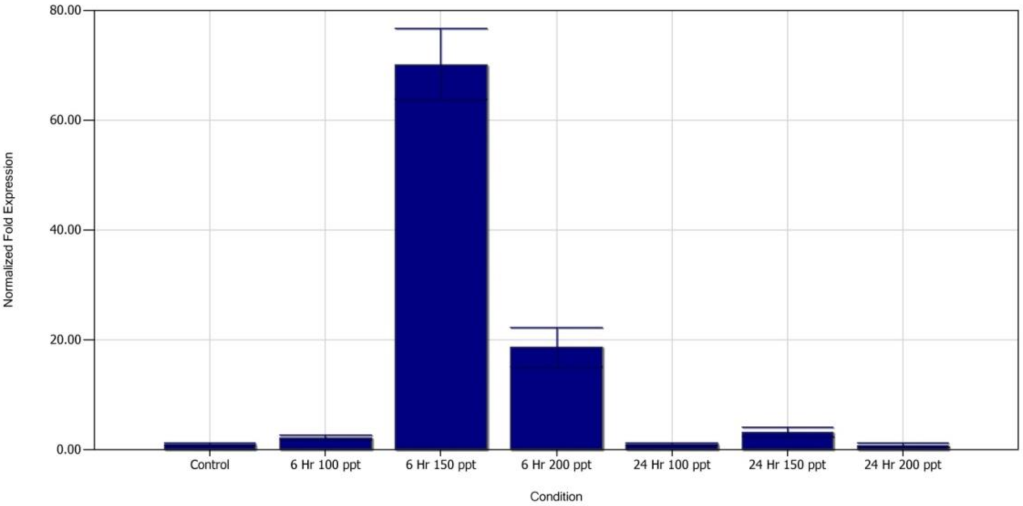


Fig.6.5 Normalized expression of ArHsp 22 gene in *Artemia* under salt stress.

6.5. Discussion

The results of the present study reveal that *Artemia* can withstand sharp increases in salinities up to 150 ppt without any mortality during the first 6 hours of exposure. But low mortality level was observed on continued incubation for 24 hours. However, at 200 ppt, low level of mortality (2.74%)

was observed up to 6 hours which showed an increasing trend, and by the 24thhour it reached 23.06 % respectively. These study show that *Artemia* can tolerate sharp increase in salinities up to 150 ppt without mortality.

Though, the results of the salinity related mortality/survivability studies reported by various workers have reported conflicting results, many of them have reported higher survivability between 100 and 180 ppt. For example, Abatzopoulos *et al*., (2006) have reported higher survival of *Artemia* in salinities of 100, 140 and 180g/l when compared to *Artemia* reared in low saline conditions viz., 35 and 50g/l. On the contrary, Agh *et al*., (2008) have reported 100% mortality of bisexual *Artemia* when reared at salinities of 150 to 200g/l. Similarly, El-Bermawi *et al.*, (2004) have also reported significantly lower survival of bisexual *Artemia* in the salinities 150 and 200 g/l when compared to lower salinities of 35, 80 and120 g/l. Browne and Wanigasekera, (2000), have reported that the survival of a parthenogenetic *Artemia* from Margherita di Savoia (Italy) increased at salinities of 60 and 180 g/l. Studies of Triantaphyllidis *et al*., (1995) showed that the survival of parthenogenetic *Artemia* of Tanggu (China) origin is not affected by the elevation of salinities up to 180 g l. Triantaphyllidis *et al*., (1995) and Stappen, (2002) have observed that the survival at high salinities is related to the efficiency of the osmoregulatory system, which was found to be reduced beyond 120g/l. According to Tackaert and Sorgeloos, (1991); Rodríguez-Almaraz *et al*., (2006), the ability of *Artemia* to withstand the hypersaline environment (100-180g/l) is a genetically imprinted ecological response, which allows them to evade competitor's/ predators in their native habitats.

According to Wang *et al.,* (2012) and Conte, (1984), upon salt stress, *Artemia* actively excrete the excess salt to the external environment with the help of different organs depending on their life cycle stage to adjust the NaCl level of the haemolymph. They tend to constantly gain NaCl and to lose water in natural condition. The low permeability of the external surface of *Artemia* may be regarded as an adaptation hindering passive diffusion of NaCl and water. Most of the permeability of the animal is across the gut epithelium, and it seems clear that in the hypersaline waters it is across this epithelium, that NaCl enters and water leaves the haemolymph. Considerable amount of permeability across the gut epithelium would be expected as various products of digestion have to be taken up and the cuticle which covers the external surface is represented by a thin peritrophic membrane in the gut (Croghan, 1957).

In the nauplii, the excretion of salt primarily depends on its transient salt gland, whereas in the juveniles and the adults it is done through the thoracic epipods that is on the appendages (Conte, 1984). Besides, in adults, the antennal glands and the maxillary glands are also known to contribute to osmoregulation (Martin, 1992). The cells of the salt gland and the epipods share morphological features consistent with their roles in electrolyte transport, including extensive membrane amplification and a rich supply of mitochondria (Conte, 1984).

The present study shows that the *Artemia* adults can tolerate sharp increase in salinity up to 150 ppt with low mortality rates, but increases with increase in salinity to 200ppt and beyond induces higher mortalities which agree with the findings of Agh*et al*., (2008). As observed by Triantaphyllidis *et al*., (1995), here also the reason for the mortalities at salinities beyond 200 ppt could be attributed to the failure of the osmoregulatory mechanisms to adjust the sudden and sharp increase in the salinity beyond a certain level.

Variations in the soluble protein associated with the rapid changes in salinity have been observed by many workers. In the present study, *Artemia* maintained at 150 and 200 ppt, showed a significant reduction in the soluble protein level up to 6 hours of incubation (16.75and 18.67 respectively) compared to the control. However, by the 24th hour the protein level at 150 ppt increased and returns back to the normal levels, while at 200 ppt the protein levels showed further reduction. On the other hand at 100ppt salinity no significant variation in soluble protein was observed during the period of incubation up to 24 hours, when compared to the control.

Various mechanisms have been suggested to be involved in the soluble protein level changes. From his studies in *Bruguiera parviflora,* Parida *et al*., (2004) have opined that the reduction in soluble protein with the rapid increase in salinity may be due to the increasing activity of acid and alkaline proteases. Protein degradation in animal tissues may vary due to the physical conformation and other properties of the protein complexes, and the large proteins are much more susceptible to degradation than smaller ones. The rate of degradation will be more in an acidic proteins than in neutral or basic proteins (Dehlinger *et al*., 1971; Dice *et al*., 1979).Qiu and Macrae, (2008) have suggested that the enhanced production of soluble proteins after long term exposure may be due to the biosynthesis of small heat shock proteins (hsp 21, hsp 22 & hsp 26) being carried out to overcome the environmental stress. Induction of heat shock genes leading to the production of heat shock proteins is the characteristic mechanism exhibited by diapausing organisms to overcome the environmental stress and for achieving the reversible dormancy.

In the present study, the total amino acid content in the *Artemia* increased significantly when exposed to salinity stress. Accumulation or over expression of nitrogen-containing compounds (proline, other amino acids, quaternary amino compounds and polyamines) and hydroxyl compound metabolites in plants, algae, fungi and bacteria under salt stress are common (Csonka, 1989; McCue and Hanson, 1990; Galinski, 1993; Potts, 1994). Short term exposure of *Artemia* to salt stress (100, 150 and 200 ppt) resulted in a significant increase in the total AA content and except in the case of 200ppt, total AA levels continued to increase significantly during long term exposure (24hour) to salt stress. According to Yoneda *et al.,* (2009) several amino acids play a significant role in regulating a variety of immune responses, including the activation of lymphocytes, NK cells, and macrophages; proliferation of lymphocytes; regulation of intracellular redox states; gene expression; and production of cytokines. The elevated expression of AA appears to be particularly important in regulating immune response following the saline stress.

Among the various classes of pigments found in nature, carotenoids are the most widespread, with important functions in various animal groups. The primary function of the carotenoids in some animals is coloration, but they have many other important roles to play, including their potential to prevent stress and enhance immunity (Von Lintig *et al*., 2004). According to Nelis *et al*., (1988), during the course of evolution, *Artemia* acquired some adaptive advantages in stress tolerance by accumulating carotenoids in its tissues and cysts in particular. Pelah *et al*., (2004) and Czygan, (1982) have reported that stress induce the biosynthesis of the secondary carotenoids astaxanthin, canthaxanthin and the total secondary carotenoids in *Chlorella zofingiensi*. Studies by Steinbrenner *et al*., (2000) and Gru¨newald *et al*., (2000) have also reported that the induced expression of carotenoid genes in the green alga *Haematococcus pluvialis* when exposed to light stress. According to Sies, (1993) the antioxidant defense system of the organism is a complex network and comprises of several enzymatic and non-enzymatic antioxidants. Among the various defense strategies, carotenoids are the most likely to be in the scavenging of two of the reactive oxygen species, single molecular oxygen, and peroxyl radicals. Soejima *et al*., (1980) reported that the canthaxanthins form the major carotenoid and they have the specific role in photoprotective function under stress.

The present study showed that short term salt stress (up to 6 hrs) reduces the carotenoid level, but as the exposure time in increased to 24 hrs, the total carotenoid level of in treatment I and II showed a marked increase, which may be, an effort to overcome the salt stress. But at 200 ppt the increase at the 24th hour was very little, and it failed to reach the normal control values.

Trehalose has the ability to stabilize and protect the membranes and proteins, allowing anhydrobiotic organisms to survive cycles of dehydration-rehydration. Hare *et al*., (1998) have reported that the trehalose functions as the compatible solute for stabilization of biological structures and serve as osmoprotectant under abiotic stress. Trehalose may also stabilize the dehydration of enzymes, proteins, and lipid membranes efficiently. Benaroudj *et al*., (2001) have reported that accumulation of trehalose enhances cellular resistance to oxygen radicals. With the reduction of the trehalose content in the cells, cellular proteins are more susceptible to covalent damage by free radicals by the increased appearance of carbonyl groups (Benaroudj *et al*., 2001). Singer and Lindquist, (1998) have observed that the ability of trehalose to reduce covalent modifications of proteins by oxidants indicates a new property that cannot be simply explained by its ability to prevent protein aggregation and to facilitate refolding of damaged polypeptides by chaperones. In the present study, when *Artemia* were subjected to salt stress, initially (up to 6 hrs) the trehalose level increased significantly in all the treatments. This is in agreement with the above observations by various workers. By 24 hrs of incubation at 200 ppt the trehalose levels decreased sharply when compared to 6 hrs of incubation, but were still higher than the controls indicating that beyond a certain salinity threshold, the animals cannot biosynthesize the required level of carotenoids which is also evident from the higher mortality rates observed in 200 ppt salinity. According to Noventa-Jordão *et al*., (1996) heat shock can induce the trehalose-6-phosphate synthase gene expression and accumulate trehalose in *Neurosporacrassa*.

The fatty acid biosynthetic pathways and profiles are widely used as biological indicators for understanding the impact of stress or experimental test factors in animals. According to Palacios *et al*., (2004) when marine organisms are exposed to low salinity stress, the level of polar fraction of fatty acids decrease while the neutral fraction increase proportionately, even if the exposure duration is short. Whereas in amphipods, saturated fatty acids in gills showed an increase when acclimatized to low saline water (Morris *et al*., 1982). In the present study, the level of total saturated fatty acids showed a marginal reduction at 6 and 24 hours of exposure to salinity stress. The lowest total saturated fatty acid was observed in treatment II (150 ppt) at the 24th hour of exposure.

In the present study, total PUFA level increased significantly in treatments I and II while it was reduced in treatment III after 6 hrs of exposure to salinity stress. However, after 24 hours, the PUFA level showed a sharp reduction in treatment I, whereas, it remained at the same level in treatment II and increased marginally in treatment III. In invertebrates, n-3 and n-6 polyunsaturated fatty acids (PUFA) are known to play a major role in many of the physiological processes (Biggers *et al*., 1992; Miller *et al*., 1996). Studies of Morohashi *et al*., (1991) revealed that the free fatty acids have an important role in the functioning of the Na+/K+-ATPase pump in *Artemia salina.* According to Towle, (1981), in addition to permeability function in gills, fatty acids present in the membrane is found to directly modulate the other osmoregulatory mechanisms such as Na+/K+-ATPase pump in gills. Robin *et al.*, (1987) have observed dynamic changes in the PUFA levels and profile in *Artemia* sp. when cultured under stressed conditions. Studies by Schauer *et al.*, (1980) revealed a correlation between survival percentage and dietary PUFA content in shrimps and fishes. In the present study a direct relationship between the PUFA content and survivability was evident in treatments I and II, but in treatment III in spite of having a high PUFA level, survivability was less when compared to other treatments.

The 22:6n3 levels increased significantly in all treatments at 6 and 24 hours of incubation when compared to control. Among the different treatments, 22: 6n3 content remained high and stable in treatment II at 6 and 24 hours of incubation. Conversion of 18: 2n-3 to 20: 5n-3 in *Artemia* is clearly evident from the fatty acid data under different salinities, where the 18: 2n-3 content was reduced with a corresponding increase in 20: 5n-3 compared to the control, at 100 ppt and 150 ppt. This indicates a higher activity of elongases and Δ5-desaturases to biosynthesize 20:5n-3 from 18:3n-3. Madigan *et al.*, (1999) have observed optimal enzymatic activity and membrane stability at high temperature or salinity conditions in certain species of thermophiles and halophiles to overcome the stress. Studies of Leray *et al.*, (1984) also showed an increase in 22:6n-3 level in fresh water Trout incubated in seawater for 24 hours.

In the present study, normalized expression of ArHsp22 gene increased marginally in treatment I while it increased sharply in treatments II and III at 6 hours of incubation. In *Artemia*, the sHSPs forms a distinctive molecular chaperone family characterized by monomers ranging from 12 to 43 kDa, and have well defined roles in preventing the irreversible protein denaturation, apoptosis resistance, modulation of actin assembly and microfilament stability and conferring thermo tolerance to the cells (Zhijun and Rae *et al.*, 2004).

Plants are also known to exhibit similar phenomenon where the salt stress induces quantitative and qualitative changes in protein content of the plant cells (Wimmer. 2003). The ArHsp22 chaperones prevent irreversible, stress induced protein denaturation, and act as the first line of defense against salt stress. Maimbo *et al*., (2007), Candido, (2002) and Guan *et al.,* (2004) are of the view that though ArHsp22 gene expression is developmentally regulated, heat shock can induce its production in adults, in contrast to ArHsp21 and p26 genes. Qiu and Macrae *et al*., (2008) reported that the mechanism by which diapausing organisms achieving reversible dormancy and increased stress tolerance is mainly by the enhanced expression of sHSP, among the different other biochemical and morphological adaptations. According to Morrow *et al.,* (2006), in *Drosophila melanogaster,* the small heat shock proteins such as Hsp22, Hsp23, Hsp26 and Hsp27 are rapidly accumulated upon heat shock though they show stage and tissue specific synthesis which is in agreement with the present findings. However in the present study, at 24 hours post-incubation, the ArHsp22 gene expression was considerably reduced when compared to the 6 hour post-incubation in all treatments.

6.6. Conclusion

Present study revealed that *Artemia* can withstand sudden increase in salinity up to 150 ppt without any mortality up to 6h of durations and with low percentage of mortality up to 24 hours of exposure. Enhanced expression of biochemical constituents such as protein, amino acid, trehalose, carotenoid and fatty acid in *Artemia* might be involved in the protection of the cell membranes and biomolecules from the salt stress. Further, the enhanced expression of ArHsp 22 might act as the first line of defense against the sudden salinity stress in *Artemia*.

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Chapter 7

Summary and Conclusion

Aquaculture is one of the fastest growing animal food producing sectors in the world, accounting for almost half of the total food fish supply. Sustainable larval production is vital to the success of aquaculture. Non availability of healthy, uniform sized, quality seeds in sufficient quantities have always been a concern in ‘capture based aquaculture’ where seeds are sourced from the wild. The above issues can be addressed through ‘hatchery based culture’ systems that can ensure the availability of quality seeds in required quantities. In hatcheries, live feeds play a key role in deciding the survival, growth and development of the larvae. Among the different live feeds, *Artemia* are widely used as the starter diet in larviculture. To lessen the dependency on natural *Artemia* sources and reduce the larval rearing expenses, identification of potential strains various formulated diets were tried as alternatives. But, due to reduced preference/acceptability by the larvae, water quality deterioration, etc. their acceptance got reduced in larviculture. In this scenario, a detailed study on native *Artemia* recourses to develop/ identify suitable superior candidate *Artemia* strain for use in larviculture is vital for the development of Indian Aquaculture industry.

In the study, an extensive survey was conducted along the East (West Bengal, Orissa, Andhra Pradesh and Tamil Nadu) and West regions (Maharashtra, Gujarat and Rajasthan) of the Indian subcontinent for the presence of *Artemia* populations. *Artemia* samples were collected from different natural hypersaline habitats along the North West (Mithapur- GMJ & Nanganvadi- GNM), South West (Tamaraikkulam-TNM & Tuticorin- TTJ) and South East (Kelambakam- CKF, Vedaranyam- VDA & Marakkanam TMM) coasts of the Indian subcontinent during 2008 to 2010. In addition, *A. franciscana* strain (SFB) from San Francisco Bay procured from commercial suppliers and strains of *Artemia tibetiana* (TBS) and *Artemia salina* (ASL) received as a gift from the *Artemia* reference Center Ghent, Belgium were also used as reference strains in the study.

- ✓ Though *A. parthenogenetica* has been reported from various geographical regions of India since 1950s, later studies revealed the gradual disappearance of *Artemia* populations from these regions. The present study reveals that all the *Artemia* populations collected during 2008-2010 were bisexual, composed of fertile males and females, indicating the absence of *A. parthenogenetica*. Further, the assessment of the reproductive pattern of the *Artemia* samples clearly showed that none of the individually reared, mature forms could alone produce any offspring confirming the total absence of parthenogenesis. On the contrary, the crossbreeding experiments clearly show that all these populations were able to cross breed, producing fully fertile offspring, thus exhibiting their breeding efficiency and fertility.
- ✓ Species level molecular analysis using ITS-1 sequence with various *Artemia* species revealed that the Indian population(s) was very close to that of the exotic *A. franciscana* with 99% homology, while other *Artemia* populations such as *A.* sinica, *A. urmiana, A. tibetiana*, *A. salina, A. persimilis* and *A. parthenogenetica* showed significant variations. Mean pairwise GD within the Indian *Artemia* populations was found to be negligible, indicating that all the Indian populations are

genetically similar and can be considered as a single species. The absence of any significant difference in the GD between the different Indian *Artemia* populations and *A. franciscana* indicate their conspecific nature. Nucleotide diversity was low in Indian *Artemia* populations indicating low genetic diversity.

- ✓ Phylogenetic analysis using the Maximum Parsimony and Neighbour Joining tree methods clearly distinguished the new world *A. franciscana* and the Indian *Artemia* populations (clade A) from the old world *A. persimilis* group (clade C).
- ✓ Present investigation reports the lack of regional endemism in different Indian *Artemia* populations of distant origins from Northeast, Southeast and Northwest regions of the Indian subcontinent indicating that these populations are comparatively young in the process of evolution. Haplotype diversity throws light into the distribution pattern of the Indian *Artemia* populations. The distribution of same haplotypes in geographically distinct habitats indicates that they have a common origin but are evolving cohesively. High singleton variation in the ITS 1 sequence as revealed by the sequence characterization is a key indicator of the population expansion while the presence of few parsimony informative sites indicate the relative absence of 'bottle necks' in the expansion of this population.
- ✓ The negative Tajima's D higher haplotype diversity, higher singleton variations, and less number of parsimony informative sites in the ITS-1 sequence of the Indian *Artemia* populations signify an excess of low frequency polymorphisms, which again indicate that the population is under expansion and/or positive selection

- ✓ The morphological and biometric observations, crossbreeding experiments, molecular analysis data, ITS-1 sequence lengths and high per cent of homology, mean pairwise GD data and phylogenetic analysis clearly proves that the *Artemia* populations collected from the various geographical regions of India are *A. franciscana* naturalized during the course of time. These observations indicate that the earlier reported *A. parthenogenetica* have been replaced by the invaded and naturalized, bisexual, *A. franciscana.*
- ✓ The disappearance of parthenogenetic *Artemia* populations the North West region of India could be ascribed to the extreme draught conditions in these habitats. In the east coast of the Indian subcontinent, the states of Andhra Pradesh, Orissa and West Bengal have numerous aquaculture farms and hatcheries where *Artemia* has been extensively used as live feed. But surprisingly, our study revealed total absence of *Artemia* in all the hypersaline water bodies of the region.
- ✓ Anthropogenic activities have aided the massive invasion of *A. franciscana* into the natural hypersaline habitats in the Indian subcontinent where it flourished and displaced the native *A. parthenogenetica* from its natural habitat. The species-ecosystem and habitat interaction has to be validated further to shed more light on this aspect. This forms the first report on the loss of the native *A. parthenogenetica* from the Indian hypersaline water bodies following the invasion of by *A. franciscana,* a species already known for invasions worldwide.
- ✓ Morphological analysis revealed the superiority of indigenous *Artemia* with respect to the cyst and nauplii size. Indigenous bisexual *Artemia*

strains showed significantly smaller cysts when compared to the earlier reported Indian *A. parthanogenetica* and other bisexual *Artemia* strains of worldwide origin. With respect to the cyst size, the Marakkanam strain appeared to be the smallest (219.0µm) while the Tamaraikulam strain had the largest cyst (236.4µm). Nauplii length of the indigenous *Artemia* strains showed marginal difference and Marakkanam strain possessed smallest (496µm) nauplii. NL of the native strains (496.0 to 514.8µm) was comparable with the various *Artemia* strains reported globally.

- ✓ Other life history estimates also showed significant differences in TDL, SDL, LMSM and LFSM values, which were also comparable with other *Artemia.*
- ✓ The Hatching percentage observed in the Indian strains during the present study ranged from 45% (TTJ) to 84.5% (GMJ) while in the commercial SFB strain it was 72.2%, indicating the better quality of the GMJ strain. Hatching percentage and cyst trehalose content of *Artemia* showed a significant positive correlation indicating that the trehalose in the cysts acts as a stabilizing agent to protect the membrane proteins, thereby helping the cysts to survive the repeated dehydration-rehydration impacts and could enhance the shelf life of the cysts.
- ✓ Soluble protein was less in cyst stage but was high and almost equal in nauplii and adult stages, making the cyst a less preferred option as a source of soluble protein. High soluble protein content in the nauplii of the indigenous *Artemia* strains (TTJ and CKF) when compared to the commercially available SFB strain further indicates their nutrient superiority.

- ✓ High levels of IAAs were observed in nauplii stage (1742.82 mg/100gm) when compared to the cyst (1146.2 mg/100gm) and adult stage (578.7 mg/100gm) of the *Artemia*. Independent of their origin and life cycle stage of the *Artemia* studied, tyrosine, methionine and histidine were less in IAAs when compared to the lysine and valine contents.
- ✓ In the case of DAAs, arginine, glutamine and aspartic acid contributed the major share in all the stages of *Artemia* while glycine, proline and cystine contributed the least. Limiting AAs such as threonine, leucine, arginine, lysine and histidine were observed in high levels in nauplii stage than the cyst and adult stages of *Artemia* which has been reported by many authors. Similarly, methionine, another limiting AA was also found to be high in the nauplii stage (36.8 to 106.4 mg/100gm) when compared to the cyst and adult stages.
- ✓ Independent of their origin, high carotenoid content was observed in adult stage than the other stages of the *Artemia* strains studied. Among the various strains of *Artemia* adults studied, TNM and TMM strains showed high carotenoid level when compared to the reference strain (SFB). These results also indicate the overall ability of *Artemia* to biosynthesize polar carotenoids from precursor molecules during the advancement of life cycle stages from cyst to adult.
- ✓ Total carotenoids in cysts (259.5 µg/gm) and nauplii (266.9 µg/gm) did not show any significant difference between the stages while significant different were observed between the strains. The naturalized strains of TNM and TMM with high carotenoid values in particular, may be

promising alternatives to the imported strains in terms of nutritional values.

- ✓ A considerable reduction in saturated fatty acid was apparent in the nauplii when compared to the cyst stages in native strains. The SFA values increased during the development from nauplii to adult stage indicating the assimilation of SFA through feed. Monounsaturated fatty acids in GMJ strain nauplii was significantly higher (45.4%) than the reference SFB strain (30.8%) and the reported *Artemia persimilis* (33.1%) and other commercial *Artemia* strains (40.6%). MUFA showed a substantial reduction in adults (2.5 fold) when compared to the nauplii and cyst stages of the *Artemia*.

- ✓ Among the different stages of the *Artemia* strains studied, high total PUFA was observed in the nauplii stage (24.0%) when compared to adult (22.1%) and cyst stages (19.1%). Indigenous strain nauplii (GMJ and TTJ) showed high 20:5*n*-3 content when compared to the reference and other strains evaluated.

- ✓ Though the reference SFB strain nauplii have a higher 18:3*n*-3 content (26.3%) it is has not been reflected in the low 20:5*n*-3 values (1.9%), indicating low biosynthesizing ability. Among the strains studied, 22:6*n*-3 was high in the nauplii of TMM, TNM, VDA and TTJ strains (0.64 to 1.71%) when compared to the reference SFB strain nauplii (0.2%), the Greek *Artemia* nauplii (0.1 to 0.3%) and the enriched *Artemia salina* nauplii (0.1%). Higher content of these essential PUFAs viz., 20:5*n*-3 and 22:6*n*-3 in the native *Artemia* strains revealed their nutritional superiority when compared to the commercially available strains.

- ✓ Morphological observations revealed the length of the freshly hatched *Artemia* nauplii within the base generation (G0) ranged from 400.0 µm to 570.0 µm with a mean value of 517.0 ± 39.8 µm.
- ✓ Mass selection was practiced in the *Artemia* nauplii to bring about change in nauplii size. Selection differential in the base generation was -25.97 and it showed variations over the generations. Selection differentials ranged from -33.17 to -8.66 µm in other generations.
- ✓ Heritability estimates of the selected *Artemia* showed generation to generation variations. Heritability was 0.99 ± 0.36 in the base generation while it varied between 0.36 and 1.64 in other generations.
- ✓ The mean nauplii length was 439.3±27.0 µm, after fifteen generations of selection, as against 517.0±39.8 µm in the base generation. A gradual decrease in nauplii length was noticed during the selection process.
- ✓ Selection gain was -2.40 µm in G1 while it reduced sharply in G2 to -9.86 µm maximum selection gain was noticed in G4 (-10.70 µm) and minimum was in G10 (-0.76 µm). Cumulative selection gain after the fifteen generations of selection was -77.67 µm.
- ✓ A reduction in cyst size was realized in the selectively bred *Artemia* strain as a correlated response. After 15 generations of selection the cyst size in the selected line (SBAS) got reduced to 212.5 ± 9.4 µm from 224.83 ± 14.81 µm in the base generation.
- ✓ Selective breeding for nauplii size reduction resulted in a correlated increase in the hatching percentage also. At the end of 15 generations of selection the hatching percentage in the selectively bred strain was 64.58% as against 54.4% in the base generation.

- ✓ Irrespective of the enrichment diet, total length (TL) of the nauplii gradually increased during the nine hour enrichment period while total width gradually reduced after the study in all the experiments. Total length and total width of the *Artemia* nauplii before the enrichment (zero hour) was 516.6 ± 61.4 µm and 164.6 ± 18.4 µm respectively.
- ✓ Among the different enrichment diets maximum nauplii growth was observed in Algamac enriched nauplii (648.3 ± 70.4 µm) followed by *P.viridis* (628.3 ± 50.7 µm), *D.inornata* (623.1 ± 55.1 µm) and *N.oculata* (614.9 ± 47.3 µm). The lowest growth rate was observed in *I. galbana* enriched nauplii (600.2 ± 72.0 µm).
- ✓ Lowest TW was observed in *N.oculata* enriched nauplii (142.5 ± 17.0 µm) followed by control (143.5 ± 12.7µm), *D.inornata* (151.8 ± 23.0 µm), *I.galabana* (151.9 ± 28 µm), Algamac (154.0 ± 18.3 µm) and *P.viridis* (156.0 ± 20.1 µm).
- ✓ After nine hours of enrichment, nauplii mortality was lowest in microalgae medium than the Algamac and un-enriched medium. Mortality percentage was 18.2±5.65% in Algamac diet which was found to be significantly higher than that in microalgae viz., *I. galbana* (3.2±1.9%), *P.viridis* (1.6±1%), *D.inornata* (0.6±0.45%) and *N.oculata* (0.5±0.37%) diet.
- ✓ The survival rate of the nauplii enriched for 1, 3, 6 and 9 hours with Algamac and microalgae, and the un-enriched nauplii, on exposure to 10, 20, 30 and 35 ppt salinities were 100%. However, higher mortality was observed when incubated at 0 ppt.

- ✓ Among the different diets, microalgae enriched *Artemia* nauplii showed significantly lower mortality percentage when compared to the Algamac enriched and un-enriched nauplii.
- ✓ After the first 1 hour exposure to 0 ppt salinity, the Algamac enriched nauplii showed high mortality of 10.6% when compared to the un-enriched nauplii and microalgae enriched nauplii. While no mortality was observed in *N.oculata* and *D.inornata* enriched nauplii, it was 1.1% in *I. galbana* and *P.viridis* enriched nauplii.
- ✓ The highest mortality at 3 hours (16.3 and 16%) was observed in algamac and un-enriched nauplii. While mortality was the lowest (6.3%) in *N. oculata* enriched nauplii, it was 9% with the other three microalgae.
- ✓ At 6 hour, though the mortality in the enriched group increased, it was lower than the control and Algamac enriched nauplii. The lowest mortality was in the *N.oculata* and *D.inornata* enriched nauplii (9.7%) followed by *I.galbana* and *P.viridis* enriched nauplii (10.4 and 11%) as compared to the 16.7% mortality in Algamac enriched and un-enriched nauplii (16.7%).
- ✓ After the 9 hour incubation at 0 ppt, highest mortality was observed in unenriched nauplii (25.2%) followed by algamac enriched nauplii (19.4%) when compared to the microalgae enriched nauplii which ranged from 10.6 to 13.1% only. Among all the enrichments, *I.galban* resulted in the lowest mortality percentage after holding at 0 ppt. salinity for 9 hours.
- ✓ Fatty acid profile of the different microalgae used for enrichment was worked out as a pre-mediated step, and it has revealed notable

differences among them. Fatty acid profile of the microalgae showed considerable variations in the level of major fatty acids. Saturated fatty acids (SFAs) were found to be low in the microalgae, *D. inornata* and *I. galbana* (23.81 and 28.95%, respectively) compared to *P. viridis* and *N. oculata* (35.16 and 38.64 %, respectively).

- ✓ Though, a gradual decline in SFAs was observed in all the microalgae enriched nauplii up to 7th hour of enrichment, it was only marginal. However, after 9th hour of enrichment, the SFA content of the nauplii showed a significant increase.
- ✓ The monounsaturated fatty acid (MUFA) content in nauplii enriched with *P. viridis, D. inornata, N.oculata* and *I.galabana* showed a gradual reduction after nine hours of enrichment (30.9%, 30.2%, 29.9% & 30.9% respectively), when compared to the unenriched nauplii (42.4%). Total MUFA of the enriched nauplii is contributed mainly by 18:1n9 (60%) followed by 16:1n 7 (30%).
- ✓ Among the microalgae sources evaluated, *I. galbana* showed the highest PUFA content (43.31%) followed by *D. inornata* (39.01%), *P. viridis* (23.41%) and *N. oculata* (19.81%). In the nauplii enriched with these microalgae, time dependent increase in the total PUFA content was clear though, the variations were only marginal. The maximum total PUFA level was reached by 7 hours following enrichment in *I. galbana*, *P. viridis* and *D. inornata* respectively and it was 29.3, 31 and 33. 9% respectively. However, by 9th hour it got reduced considerably to 18.5, 22.1 and 23.4% respectively.

- ✓ The maximum level of 20:5n3 with *N. oculata* (7.81%) and with *P.viridis* (7.58) were comparable, but in case of the former it was reached in 5 hours as against 7 hours in the later.
- ✓ In case of 20:4n6 and 18:3n6 an incremental trend was visible wherein the maximum level was reached at 5th hour of enrichment with *N. oculata* (2.15 and 1.56% respectively), while in all others maximum was reached after 7th hour of enrichment. Total n3 PUFAs were high in *N. oculata* enriched nauplii, and it ranged from 8.52 (during 1st hour of enrichment) to 13% (during 5th hour of enrichment).
- ✓ The present study shows that *Artemia* adults can tolerate sharp increase in salinity up to 150 ppt with low mortality rates, but increases in salinity beyond 200ppt induces higher mortalities.
- ✓ In the case of treatments I (100 ppt) and II (150 ppt), mortalities started appearing after the first 6 hours of incubation and continued up to 24 hours post incubation (2.84±1.05 and 2.89±1.44 respectively). Treatment III (200 ppt) showed the highest mortality with 2.74 % mortality up to 6th hour which later on further increased sharply (23.06 %) up to 24th hour of incubation.
- ✓ Except in treatment I at 6 hours post incubation, a significant reduction in the soluble protein levels was observed in all the treatments. At 24 hours of incubation, the soluble protein level showed a significant increase in treatment II, while a significant reduction was observed in treatment III.
- ✓ At 6 hours post incubation, the total carotenoid content showed a significant reduction in treatments I, II and III when compared to the control. At 24 hours post incubation, the total carotenoid content

showed a significant increase in treatments I and II when compared to the control. While in treatment III, it showed a marginal increase when compared to the values at 6 hour post incubation, but failed to reach the normal control values at 24 hours post incubation.

- ✓ The trehalose content at 6 hours post-incubation increased significantly in treatments I, II and III when compared to the control. At 24 hours post-incubation, the values showed a significant reduction when compared to the previous observations at 6 hours but were still higher than the control.
- ✓ At 6 hours post-incubation the level of total saturated fatty acids showed a marginal reduction in treatments I and III while treatment II showed a significant reduction (35.84%) when compared to the control. While at 24 hours post-incubation, the total saturated fatty acid levels further reduced in treatments I, II and III when compared to the control.
- ✓ Monounsaturated fatty acid (MUFAs) values at 6 hours post-incubation varied in all the three different treatments with treatment I showing a significant reduction, treatment II remaining unchanged and treatment III showing a significant increase when compared to the control.
- ✓ Except in the case of treatment I, total monounsaturated fatty acid content decreased when compared to the values at 6 hours. But when compared to the control except in treatment II, monounsaturated fatty acid content showed an increase at 24 hours post-incubation.
- ✓ At 6 hours post-incubation, total PUFA content showed a significant increase in treatment I and II while there was a significant reduction in treatment III when compared to the control. PUFA content at 24 hours post-incubation showed a sharp reduction in treatment I when compared

to the values at 6 hours and control while in treatment II the values remained same as that at 6 hours but was still significantly higher than the control. In treatment III the PUFA content showed a marginal increase when compared to the values at 6 hour and control.

- ✓ Except in treatment III, the 20:5n3 content increased in treatments I and II when compared to the control at 6 hours. At 24 hours post-incubation, the trend reversed with treatment I showing a considerable reduction followed by treatment II with a marginal reduction when compared to the control while treatment III exhibited a significant increase when compared to the control and 6 hour incubation.
- ✓ Significant increase in 22: 6n 3 levels was observed in all the three treatments at 6 hours of incubation when compared to the control. At 24 hours marginal reduction in 22: 6n 3 levels was observed in treatment I when compared to values at 6 hours and control. In treatment II the values showed a marginal reduction when compared to the values at 6 hours but still remained high when compared to the control. In treatment III the 22: 6n 3 content showed a marginal increase when compared to the values at 6 hours and control.
- ✓ When exposed to high salinity regimes for 6 and 24 hours, the total amino acid content showed a steady increase. At 6 hour post-incubation, the total amino acid content increased significantly in treatments I, II and III when compared to the control. At 24 hour post-incubation, the total amino acid content was significantly high in treatments I and II when compared to the control while in treatment III a marginal reduction was observed when compared to the value at 6 hours, but was

still higher than the control. The same pattern was observed for DAA and IAA values in *Artemia* subjected to salinity stress.

- ✓ Melt curve dissociation analysis showed specific single peaks for ArHsp22, β actin and GAPDH genes in real time RT-PCR analysis indicating the specificity of the reaction. The normalized expression of ArHsp 22 gene in *Artemia* showed a gradual increase in treatment I at 6 hours of incubation. While in treatments II and III the expression showed a sharp increase and recorded a maximum expression in treatment II at 6 hours of incubation. However at 24 hours post-incubation, the ArHsp22 gene expression was considerably reduced when compared to the 6 hour post-incubation . Irrespective of the incubation period, the normalized expression of ArHsp22 gene was highest in treatment II at 6 hours of incubation.

Conclusions

The present investigation sheds light on the massive invasion and naturalization of the exotic *Artemia franciscana* species into the Indian saline ecosystems, displacing the native parthenogenetic *Artemia*. Their cyst size and nauplii size of these naturalized geographic strains were comparable with the commercial strain of *A. franciscana* and even better in some of the geographic strains. The exceptional phenotypic plasticity, broad tolerance to diverse environments and the breeding pattern of the alien *A. franciscana* species might have facilitated its invasion in to the Indian subcontinent. This forms the first report on the displacement of parthenogenetic *Artemia* by the invading *A. franciscana*.

The present investigation also provides valuable insights into the nutritional variations in the cyst, nauplii, and adult stages of these indigenous

Artemia strains from the hypersaline habitats. The study revealed the superior nutrient quality in terms of soluble protein, IAA, DAA, carotenoid and fatty acids in the these indigenous *Artemia* strains when compared to the widely used commercial strains. Such indigenous strains with higher nutritional profile can either be used directly as live feed through large scale farming, or may be explored as base strains for selective breeding programmes to develop better strains.

The present study has established the effectiveness of selective breeding in bringing reduction in nauplii size. The study reports the reduction in nauplii length by 14.9% as the direct response to fifteen generations of mass selection for nauplii size reduction in indigenous *Artemia*. Reduction of cyst size (5%) and increase in the hatching percentage (10 %) were also realized as correlated response. The selectively bred *Artemia* strain developed from the present work has small sized nauplii and cyst, and high hatching efficiency making it a promising candidate strain for larviculture. Further studies are required to validate the performance of the strain in field conditions.

Present study explored the suitability of using live microalgae as enrichment diet for *Artemia* nauplii. Microalgae are superior enrichment diet for *Artemia* nauplii due to the higher survival rate, nutritional content and salinity tolerance in the enriched nauplii as compared to the commercial enrichment diet.

The effect of salinity stress on *Artemia* was studied. The study revealed that *Artemia* can withstand sudden increase in salinity up to 150 ppt without any mortality up to 6h of durations and with low percentage of mortality up to 24 hours of exposure. Enhanced expression of biochemical constituents such as protein, amino acid, trehalose, carotenoid and fatty acid have been noticed in

Artemia following exposure to salinity stress, and might be involved in the protection of the cell membranes and biomolecules from the salt stress. Further, enhanced expression of the small heat shock protein ArHsp 22 was also detected, which might be functioning as the first line of defense against the sudden salinity stress in *Artemia*.

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Publications Index

Sl no	Title		Status
	PEER REVIEWED JOURNAL ARTICLES		
1.	**P. A. Vikas,** N. K. Sajeshkumar, P. C. Thomas,Kajal Chakraborty, K. K. Vijayan. Aquaculture related invasion of the exotic Artemia franciscana and displacement of the autochthonous *Artemia* populations from the hypersaline habitats of India. **Hydrobiologia** (2012) 684:129–142.	:	Published
2.	**Vikas, P. A**., Chakraborty, K., Sajeshkumar, N. K., Thomas, P. C., Sanil, N. K., Vijayan, K. K. Unraveling the effects of live microalgal enrichment on *Artemia* nauplii (2012). **Indian Journal of Fisheries** 59(4), 111–121.	:	Published
3.	**Vikas P.A**, Kajal Chakraborty, Sajeshkumar N.K, Thomas P.C, Sanil, N.K, Vijayan K.K. Quality evaluation of six allochthonous *Artemia franciscana* strains from the hypersaline habitats of Indian subcontinent. (2013). **Journal of Applied Aquaculture.**	:	In Press
4.	**Vikas P.A,** Sajeshkumar N.K, Thomas P.C, Kajal Chakraborty, Jayasankar J, Vijayan K.K. Selective breeding for nauplii size reduction in the naturalized Artemia franciscana from Indian Salinas.	:	Under Review
5.	**Vikas P.A,** Sajeshkumar N.K, KajalChakraborty, Sanil N.K, Thomas P.C and Vijayan K.K. Analysis of the genetic and biochemical adaptations of Arthropods to withstand salt stress in hypersaline habitats; A case study.	:	Under Review
	PATENT		
6.	Thomas P.C, **Vikas P.A***, Sajeshkumar N.K, Kajal Chakraborty, and Vijayan K.K. A process to prepare naturalized Artemia franciscana from Indian subcontinent with high docosahexaenoic acid and trehalose for aquaculture applications. **2063/CHE/2010**		Patent Filed
	GENBANK ACCESSIONS		
7.	**Vikas,P.A.,** Sajeshkumar,N.K., Thomas,P.C., Chakraborty,K. and Vijayan,K.K. Twenty seven Internal Transcribed Spacer Sequence 1 (ITS-1) of seven geographic strains from India and two specimens of *A. franciscana* reference strain were sequenced and deposited. **NCBI Accession**: GU323289, GU323290, GU323291, GU323292, GU323293, GU323294, GU323295, GU323296, GU323297, GU323298, GU323299, GU323300, GU323301, GU323302, GU323303, GU323304, GU323305, GU323306, GU323307, GU323308, GU323309, GU323310, GU323311, GU323312, GU323313, GU323314, GU323315, GU323316, GU323317.	:	Published
	PAPERS IN INTERNATIONAL CONFERENCES		
8.	**Vikas P.A***, Sajeshkumar N.K, Kajal Chakraborty, Thomas P.C and Vijayan K.K. Analysis of the genetic and biochemical adaptations of Arthropods to withstand salt stress in hypersaline habitats; A case study. World Conference on Marine	:	Presented

	Biodiversity (WCMB), 26-30 September 2011 at **Aberdeen, Scotland (UK).** September 24 to 30, 2011.		
9.	**Vikas P.A***, Sajeshkumar N.K, Kajal Chakraborty, Thomas P.C and Vijayan K.K. Analysis of the real time expression of heat shock protein 22 gene (hsp22) and trehalose on stress resistance in the cryptobiotic A*rtemia* and it's life stages. Asian Pacific Aquaculture 2011. January 17-20, 2011.	:	Presented
10.	Sajeshkumar N.K, **Vikas.P.A**, Thomas.P.C, Paulton. M.P and Vijayan.K.K.Identification and characterisation of long chain fatty acid elongase gene in the brine shrimp *Artemia*. Asian Pacific Aquaculture 2011. January 17-20, 2011.	:	Presented
11.	Sajeshkumar N.K, **Vikas.P.A**, Thomas.P.C, Paulton. M.P and Vijayan.K.K. Molecular Identification of Indian *Artemia*. Asian Pacific Aquaculture 2011. January 17-20, 2011.	:	Presented
12.	**P.A.Vikas.** Kajal Chakraborty, N.K.Sajeshkumar, P.C.Thomas and K.K.Vijayan. Microalgal diet induced changes in fatty acid composition of *Artemia* collected from hypersaline habitats of India for use in mariculture. 7th Asia Pacific Conference on Algal Biotechnology, University of Delhi, 1st to 4th Dec. 2009.	:	Presented
13.	**Vikas P.A**, Sajeshkumar N.K, Thomas P.C, Kajal Chakraborty, Vijayan K.K. Extensive use of alien Artemia strains in larviculture industry in India: the impact on native Artemia biotopes. 9th Indian Fisheries Forum, Chennai, 2011 December 19 to 23.	:	Presented
14.	Sajeshkumar.N.K. **Vikas.P.A**., Thomas.P.C., Kajal Chakraborty., Jayasankar.J., Vijayan.K.K. Correlated response in PUFA profile of *Artemia franciscana* from the Indian Salinas Following quantitative genetic manipulation for nauplii size reduction. 9th Indian Fisheries Forum, Chennai, 2011 December 19 to 23.		Presented

Aquaculture related invasion of the exotic Artemia franciscana *and displacement of the autochthonous* Artemia *populations from the hypersaline habitats of India*

**P. A. Vikas, N. K. Sajeshkumar,
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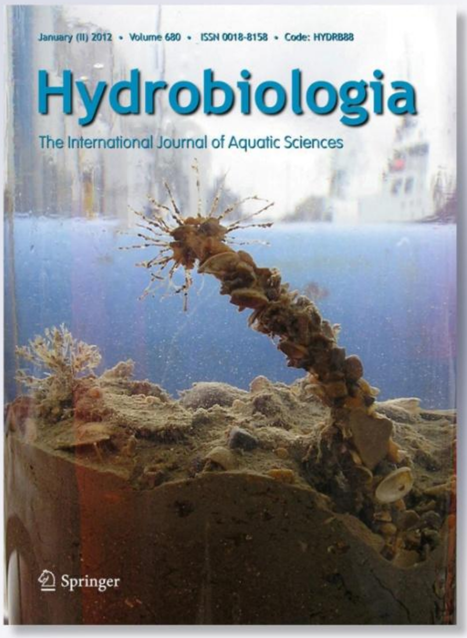

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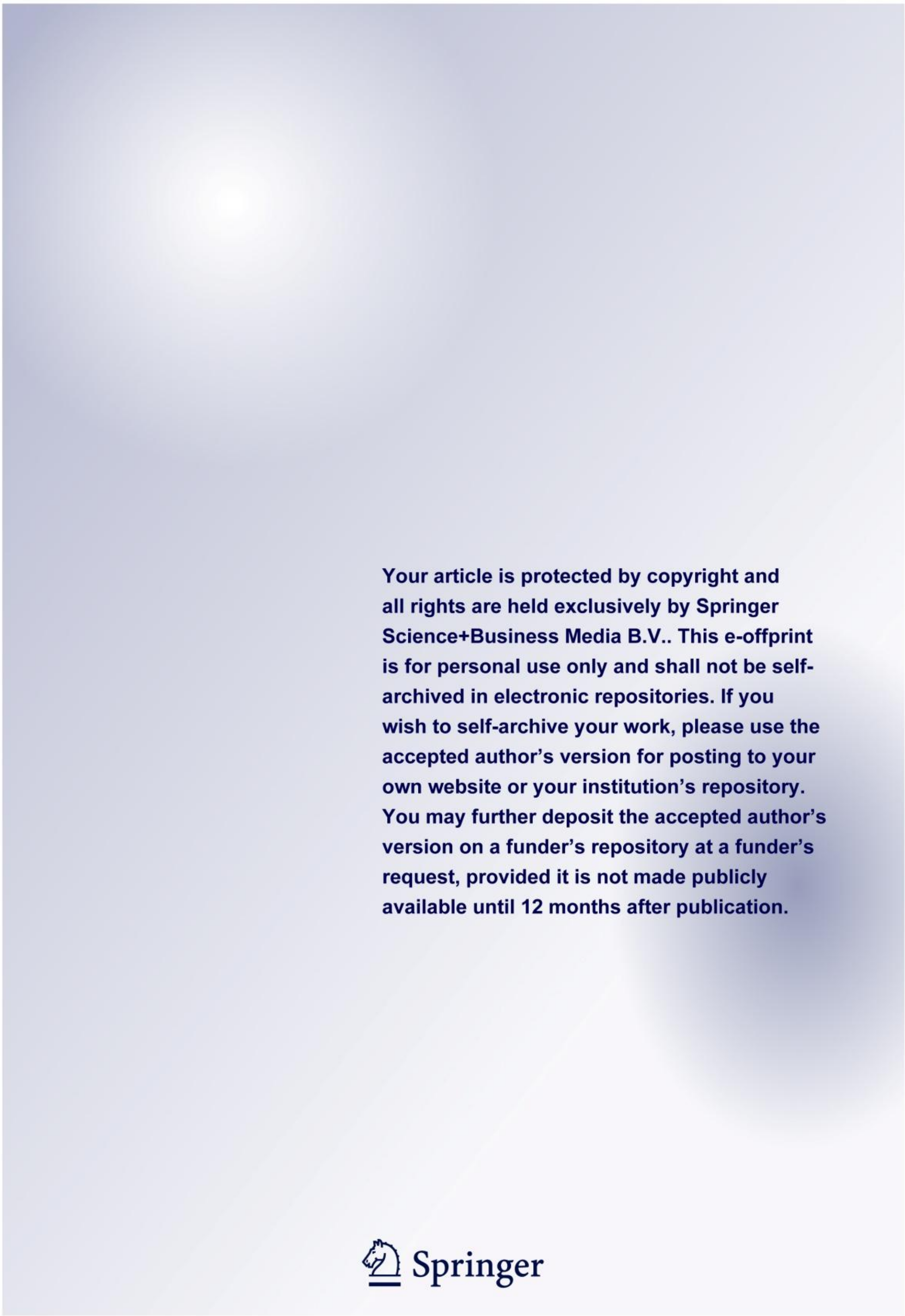

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PRIMARY RESEARCH PAPER

Aquaculture related invasion of the exotic *Artemia franciscana* and displacement of the autochthonous *Artemia* populations from the hypersaline habitats of India

**P. A. Vikas · N. K. Sajeshkumar · P. C. Thomas ·
Kajal Chakraborty · K. K. Vijayan**

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Abstract Autochthonous parthenogentic *Artemia* populations have been reported from Indian hypersaline habitats since 1950s. Exotic *Artemia franciscana* was imported and introduced into India as live food for aquaculture since the early eighties. To assess the present status of the *Artemia* populations and the possibility of invasion by the introduced *A. franciscana* in Indian Salinas, an extensive study was conducted using conventional and molecular approaches. Morphological and biometric observations, crossbreeding experiments and molecular and phylogenetic analysis using Internally Transcribed Spacer-1 sequence revealed the extensive presence of alien, sexual *A. franciscana* populations in various hypersaline areas. Individual culture experiments and crossbreeding studies further confirmed the absence of autochthonous parthenogentic *Artemia* populations. Lack of regional endemism in populations of distant origins was evident, indicating that the invaded populations have naturalized and are in the process of evolution. This forms the first report of invasion by *A. franciscana* in hypersaline habitats of Indian subcontinent and further studies are required to assess the biological implications of this invasion.

Keywords *Artemia franciscana* · Invasion · Molecular phylogeny · Naturalization

Introduction

The principal components of human-induced alterations to aquatic ecosystems are the introduction of exotic organisms and loss/alteration of habitat resulting from aquaculture and related activities. An exotic invasive species is one, which if introduced, will become established and inflict damage on the biodiversity, ecosystem integrity, agriculture, fisheries, and public health (Allendorf & Lundquist, 2003). Invasive species usually have an edge during competition with the native species for food and space and often remain at low levels for an extended period of time before being fully established. They slowly colonize the habitats, and become adapted to the native habitats and eventually replace the native species in the course of time (Ruiz et al., 1999). Many native aquatic species are threatened by competition and predation from

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P. A. Vikas (✉) · N. K. Sajeshkumar ·
P. C. Thomas · K. Chakraborty · K. K. Vijayan
Genetics and Genomics Section, Marine Biotechnology
Division (MBTD), Central Marine Fisheries Research
Institute (CMFRI), Post Box No. 1603,
Ernakulam North P.O., Cochin 682018, Kerala, India
e-mail: vikaspattath@gmail.com

invaders, and invasive species are a leading cause of animal extinctions, resulting in ecosystem modification and loss of biodiversity (Mooney & Cleland, 2001; Gurevitch & Padilla, 2004; Vijayan et al., 2004; Clavero & García-Berthou, 2005; Vijayan and Syda Rao, 2009). Apart from their substantial economic and environmental impacts, invasive species may alter the evolutionary trajectory of natives through competition, displacement, hybridization, and even extinction (Sakai et al., 2001) which is especially detrimental for a variety of continental aquatic invertebrates with dormant life stages and similar morphology (Muñoz et al., 2010).

The brine shrimp *Artemia* (Branchiopoda, Anostraca) is widely distributed in salt pans, inland salt lakes, and hypersaline water bodies throughout the world (Royan et al., 1970; Van Stappen, 2002). It comprises a group of sexual and parthenogenetic species which probably diverged five to six million years ago from an ancestral form living in the Mediterranean area (Triantaphyllidis et al., 1998; Abatzopoulos et al., 2002; Muñoz et al., 2010). *Artemia parthenogenetica* is distributed over a vast geographic area, including the continents Australia, Europe, Asia, and Africa (Gajardo et al., 2002; McMaster et al., 2007). In contrast, sexual *Artemia* species are geographically restricted (Muñoz et al., 2010). In India, the occurrence of native parthenogentic *Artemia* populations was first reported from Vadala, Mumbai (Kulkarni, 1953). Subsequently, many researchers reported the occurrence of this strain from various regions of the Indian subcontinent (Baid, 1968; Royan et al., 1970; Achari, 1971; Royan, 1979; Bhargava & Alam, 1980; Basil et al., 1987; Kulasekarapandian et al., 1992; John, 1994).

Artemia franciscana, a non-native species in India, has been used as live feed for over 85% of the fin fish and shell fish larvae (Chakraborty et al., 2010). Its introduction started in early 1980s as live feed to use in finfish and crustacean hatcheries and commercial culture ventures (Kulasekarapandian et al., 1995). During the last three decades the Indian aquaculture industry have been importing exotic *A. franciscana* strains mainly from San Francisco Bay (California), Great Salt Lake (Utah) in the United States, and salt lakes from Saskatchewan in Canada (CMFRI, 2006). The first recorded deliberate introduction of *A. franciscana* for aquaculture purpose was on Pacific Island in Brazil in the 1970s (Van Stappen, 2002). Amat et al. (2005) have remarked *A. franciscana* as an exotic invasive species in the western Mediterranean countries, including the Atlantic shore salterns in Portugal and southwest Spain, France, as well as in the north of Morocco. Presently, *A. franciscana* is considered as an invasive species throughout the world as they compete with the native brine shrimp populations due to its superior adaptive capabilities (Amat et al., 1991, 2007; Ruebhart et al., 2008).

The present study aimed to (a) characterize the *Artemia* populations in the Indian subcontinent, (b) investigate the extend of invasion and naturalization of exotic *A. franciscana* in Indian salinas, and (c) assess the displacement of the native parthenogenetic *Artemia* from its natural habitats. Hou et al. (2006) have observed that variations in morphometric characters and the genetic variability of internal transcribed spacer sequence can be used as tools in taxonomic and phylogenetic studies. During the present study, morphometric analysis, crossbreeding experiments and phylogenetic analyses, using ITS-1 sequences, were used to characterize the *Artemia* populations collected from various hypersaline habitats of India.

Materials and methods

Sampling

Artemia samples were collected from different natural hypersaline habitats along the North West, South West, and South East coasts of the Indian subcontinent during 2008 to 2009 (Table 1; Fig. 1). Adult individuals were collected using scoop nets (0.1 and 1 mm mesh) and packed with oxygen in polyethylene bags; cysts were packed in polyethylene bags with hypersaline brine (250‰) and transported to the wet laboratory of CMFRI (Cochin) for further studies. Adults were maintained in acrylic tanks (50 l), while cysts were cleaned and processed by biphasic flotation method (Sorgeloos et al., 1986) and kept at 4°C until analysis. In addition, *A. franciscana* strain (SFB) from San Francisco Bay procured from commercial suppliers and strains of *Artemia tibetiana* (TBS) and *Artemia salina* (ASL) received as a gift from the *Artemia* reference centre Ghent, Belgium, were also used in the study.

Table 1 List of the collected *Artemia* population, origin, date of collection, nature of sample, species name, GenBank Accession numbers, and the status

Individual ID	Collection site	Date of collection	Geographical coordinates	Nature of sample	Identified species	GenBank accession no.	Previous reports
CKF 008	Kelambakam, Tamil Nadu, India	02-'08	12°47′N–80°13′E	*Artemia* Live & Cyst	*A. franciscana*	GU323289	Kulasekarapandian et al. (1992)
CKF 003						GU323317	
GMJ 010	Mithapur, Gujarat, India	06-'08	22°24′N–69°01′E	*Artemia* Live & Cyst	*A. franciscana*	GU323290	Royan (1979)
GMJ 006						GU323291	
GNM 001	Nanganvadi, Gujarat, India	03-'09	23°07′N–70°41′E	*Artemia* Live	*A. franciscana*	GU323292	Present study
GNM 002						GU323293	
GNM 003						GU323294	
GNM 004						GU323295	
GNM 005						GU323296	
GNM 006						GU323297	
SFB 004	San Francisco Bay, USA	2008	37°40′N–122°17′E	*Artemia* Cyst	*A. franciscana*	GU323298	
SFB 005						GU323299	
TMM 001	Marakkanam, Tamil Nadu, India	03-'09	12°12′N–79°56′E	*Artemia* Live & Cyst	*A. franciscana*	GU323300	Present study
TMM 003						GU323301	
TNM 001	Thamaraikulam, Nagercoil, India	02-'09	08°06′N–77°29′E	*Artemia* Cyst	*A. franciscana*	GU323302	John (1994)
TNM 002						GU323303	
TNM 003						GU323304	
TNM 004						GU323305	
TNM 005						GU323306	
TNM 006						GU323307	
TTJ 002	Tuticorin, Tamil Nadu, India	06-'08	08°46′N–78°08′E	*Artemia* Live & Cyst	*A. franciscana*	GU323308	Royan et al. (1970), Achari (1971)
TTJ 004						GU323309	
TTJ 006						GU323310	
TTJ 008						GU323311	
TTJ 009						GU323312	
VDA 003	Vedaranyam, Tamil Nadu, India	04-'08	10°22′N–79°51′E	*Artemia* Live & Cyst	*A. franciscana*	GU323313	Basil et al. (1987)
VDA 007						GU323314	
VDA 010						GU323315	
VDA 005						GU323316	

Fig. 1 Map showing the survey sites in India. *Black circles with white letters* indicate the presence and transparent pentagons with *black letters* indicate the absence of *Artemia* in that location

Artemia rearing

Artemia cysts (1 g/l) were hydrated and hatched as described by Vanhaecke & Sorgeloos (1980). Freshly hatched *Artemia* nauplii were harvested and stocked in acrylic tanks (40 l) for further growth at 70‰ salinity at 25 ± 1.5°C. Nauplii were fed with microalgae, *Isochrysis galbana* (30×10^4 cells ml^{-1}) ad libitum from a microalgae culture maintained at the laboratory under mild aeration at a 12D:12L photoperiod.

Morphological observations

Randomly collected adult *Artemia* individuals from each population were fixed in formaldehyde (10%) and observed for morphological characters under a stereozoom microscope (Leica, Wetzlar, Germany).

Cyst biometry

Artemia cysts were incubated in fresh water at 28°C for 2 h for hydration and fixed with Lugol's Iodine solution. The maximum diameter (μm) of the 500 representative cyst samples from each location was determined using a microscope under 10× magnification (Leica, Wetzlar, Germany) attached with DIGI EYE 330 camera with the Dewinter software Biowizard.

Reproductive pattern

For assessing the reproductive pattern of the collected *Artemia* stocks (CKF, VDA, TTJ, TMM, GMJ, GNM, TNM, SFB), individual culture experiments were conducted. From each group, 500 *Artemia* juveniles were reared individually in plastic bottles (250 ml). The animals were maintained and observed daily until death (3–4 months) as per the standard rearing methods.

Crossbreeding experiments

Experiments were conducted to test the breeding efficiency and reproductive performance of the collected populations. Cysts of the CKF, VDA, TTJ, GMJ, and reference SFB strains were hatched out following the standard procedure (Vanhaecke & Sorgeloos, 1980) and grown for 10 days in separate tanks. Juveniles were sexually segregated and maintained in plastic containers (3 l) for 7 days prior to the experiment. Mature males (500 individuals) from each group were maintained with mature females (500 individuals) from every other group in 25 separate culture tanks (5 l) until precopulatory pairs were formed. From each tank, 25 mating pairs were selected and maintained separately in 250 ml containers. Each container was observed daily, the cysts and hatched nauplii were counted and morphometric measurements made. To assess the phenotypic variations in the F2 progenies, the nauplii were reared until maturity and life history traits such as first day length (FDL), third day length (TDL), sixth day length (SDL), length of male at sexual maturity (LMS), and length of female at sexual maturity (LFS) were recorded. Mean, standard deviation, and one-way ANOVA was computed using SPSS 13.0 program (SPSS Software, Chicago, USA).

Molecular characterization

Total genomic DNA of adult *Artemia* was isolated following the procedure described by Sambrook et al. (1989). The isolated DNA were quantified in a BioPhotometer (Eppendorf, Hamburg, Germany) and stored at −20°C until analyzed. The 18S ribosomal RNA gene, partial sequence; ITS 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence, was amplified using PTF (Tang et al., 2003) and PTR (Hou et al., 2006) primers. PCR amplifications were performed on a PTC-100® Peltier thermal cycler (Perkin Elmer Gene Amp PCR system 2400, USA). The total reaction volume (25 μl) consisted of 1× assay buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) supplemented with $MgCl_2$ (1.5 mM) (Sigma Aldrich), template DNA (20 ng), 10 pM of PTF and PTR primers, 200 μM of each dNTPs (Finzyme, USA), and 1.5 U of Taq DNA polymerase (Sigma Aldrich). Thermal cycling conditions were as follows: initial denaturation at 95°C for 4 min, 35 cycles of 94°C 30 s, 59°C 45 s, 72°C 45 s and a final extension of 7 min at 72°C. The amplified PCR products were resolved through agarose gel electrophoresis (1.2%) and documented using gel documentation system and cleaned up using a PCR product purification kit (HiMedia, India). The purified PCR products were sequenced by dideoxy chain-termination method (Sanger et al., 1977) using the AB 3730 XL capillary sequencer (Applied Biosystems, USA).

The sequences were analyzed using Gene Tool Lite software (Syngene). Sequences were aligned using the CLUSTAL-W algorithm (Thompson et al., 1994) in Bioedit 7.0 (DNA Sequence Analysis Software 224 package). For constructing the phylogenetic tree, 18S ribosomal RNA gene, partial sequence; ITS 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence from the present study (GenBank Accession Nos. GU323289–GU323317) and other reference sequences taken from GenBank database (Accession Nos., FJ004942–FJ004946, FJ004923–FJ004924, DQ084193–DQ08419, DQ084193, DQ084198, DQ069927–DQ069928, DQ201308–DQ201309, DQ201269–DQ201270, FJ004929, and DQ201276) were used. To root the phylogeny, Fairy Shrimp (*Streptocephalus proboscideus)* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence (AY519840) were used (Baxevanis et al., 2006). Pairwise genetic distances (GDs) between the collected samples were calculated based on Kimura 2 parameter model (Tamura et al., 2007). Phylogenetic analysis was carried out using maximum parsimony (MP) and neighbor joining (NJ) method with the software MEGA version 4.1. The heftiness of the phylogenetic tree branches was assessed with 1,000 bootstrap pseudo-replicates. Nucleotide sequence characteristics, nucleotide diversity, and haplotype diversity were estimated using DNAsp version 4.0 (Rozas et al., 2003). Principal Coordinate Analysis (PCoA) was performed using the software PAST (Palaeontological Statistics) (Hammer et al., 2001).

Results

The survey revealed that *Artemia* populations were present in the states of Gujarat (Mithapur and Nanganvadi) and Tamil Nadu (Tamaraikulam, Tuticorin, Vedaranyam, Kelambakkam, and Marakanam), while they were not observed in Rajasthan (Sambhar and Didhwana), Maharashtra (Vadala, Uran, and Thane), Andhra Pradesh (Nellur, Ramatheertham, and Iskapalli), Orissa (Ganjam, Humma, Sumadi, Suran, and Model salt farm), and West Bengal (Contai/Kanthi, Digha, and Sankarpur) (Fig. 1).

Morphological observations

Morphological observations revealed the presence of males with large second antennae (claspers) and distal penis and females with brood pouches in all the collected *Artemia* populations.

Cyst biometry

Cyst diameters exhibited considerable variation among the different *Artemia* populations. Cyst diameter of the Marakanam population (TMM) appeared to be the smallest (219.63 ± 10.99 μm) while that of Tamaraikkulam TNM (236.37 ± 19.00) appeared to be the largest. The reference strains received from the *Artemia* reference centre, Gent, Belgium viz., *A. tibetiana* (TBS) and *A. salina* (ASL) possessed the largest cyst size (319.39 ± 24.74, 261.72 ± 17.35 μm, respectively) while the commercial *A. franciscana* cyst size was closer to the Indian samples. Mean values of the cyst diameter showed significant differences between Indian *Artemia* populations and the reference strains, TBS and ASL except SFB ($P < 0.05$) (Fig. 2).

Reproductive pattern

Artemia juveniles reared in individual containers matured and after 3–4 months of growth, died without producing any nauplii/cyst, indicating the absence of parthenogenesis in the collected Indian *Artemia* populations.

Crossbreeding experiments

The crossing of 25 combinations of the *Artemia* populations produced nauplii and cysts. The rates of reproductive output and cyst/nauplii numbers showed variations. No infertile pairs were observed among the different crosses, indicating the absence of any reproductive barrier among the Indian populations as well as with the exotic *A. franciscana*. The entire crossbred progeny matured and produced F2 generations with different degrees of reproductive output, which further confirmed the fertility of the progeny.

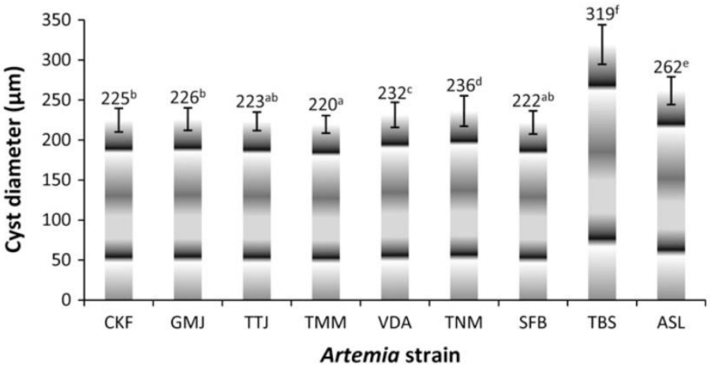


Fig. 2 Mean cyst diameter (μm) of non-decapsulated cysts of Indian and reference *Artemia* populations with standard deviation and one-way ANOVA. *Superscripts with different alphabets* indicates the significant difference between the *Artemia* populations ($P < 0.05$)

Different crossbred F2 progenies showed significant difference in first day length, while no significant differences were noted at the third day length, sixth day length, and length at sexual maturity ($P < 0.05$).

Molecular characterization

The alignment of sequences generated revealed a matrix of 1430 bp. The G+C content of the sequences ranged from 29.4 to 18.4% with an average of 23.9%. The size of the ITS1 sequence varied from 992 to 924 bp, matching that of *A. franciscana* (923–924 bp) and also showed 99% homology. Pairwise GD between the Indian *Artemia* populations from all the seven geographical locations appeared to be very low and ranged from 0 to 0.01. PCoA clearly distinguished the genetically similar Indian *Artemia* populations and the reference *A. franciscana* into a single cluster and the genetically distant *A. persimilis* (0.53–0.554), *A. salina* (0.18–0.228), and *A. sinica* (0.1418–0.137) into separate clusters (Fig. 3).

The topologies of the MP and NJ phylogenetic trees of different *Artemia* species were found to be congruent and hence the NJ tree is not illustrated. Both trees revealed three main clades (A, B, and C) with moderate to high bootstrap values (>79). In the MP tree, Indian *Artemia* populations and *A. franciscana* were grouped under clade A with a bootstrap value of 99. Clade B is subdivided into two groups, a unique *A. salina* group and a mixed group consisting of *A. sinica, A. urmiana, A. kazakhastan, A. tibetiana, Artemia* sp. China, and *A. parthenogenetica* formed with a bootstrap value of 79, while individuals of *A. persimilis* group formed an independent clade C with a bootstrap value of 99 (Fig. 4).

Sequence analysis revealed high polymorphism among the Indian *Artemia* populations. Twenty-two haplotypes were observed out of the 29 *Artemia* individuals analyzed from the different populations (Fig. 5). Haplotype A was present in three geographically distinct habitats in the Northwest (GNM) and Southeast regions (CKF and TTJ) of the Indian

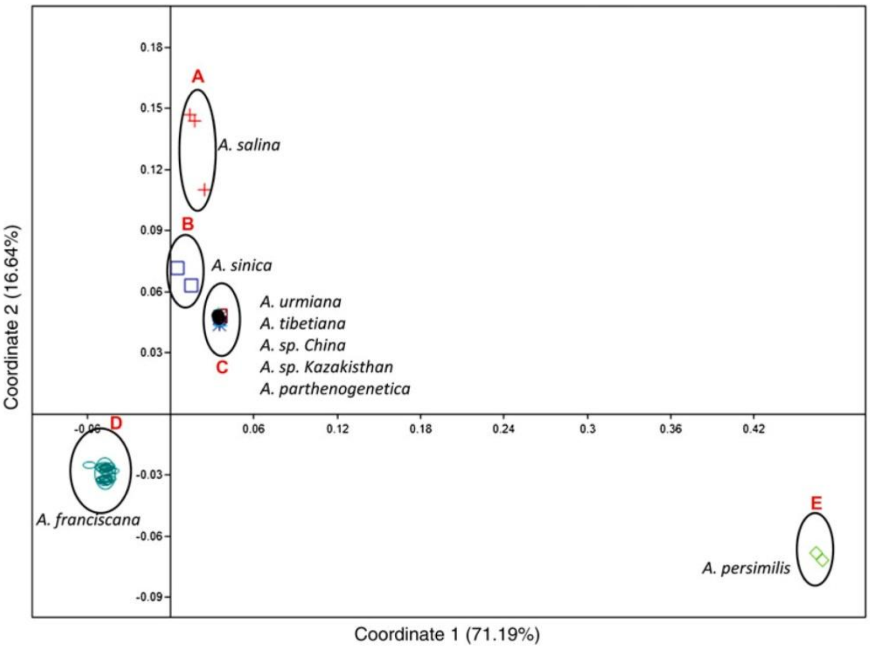
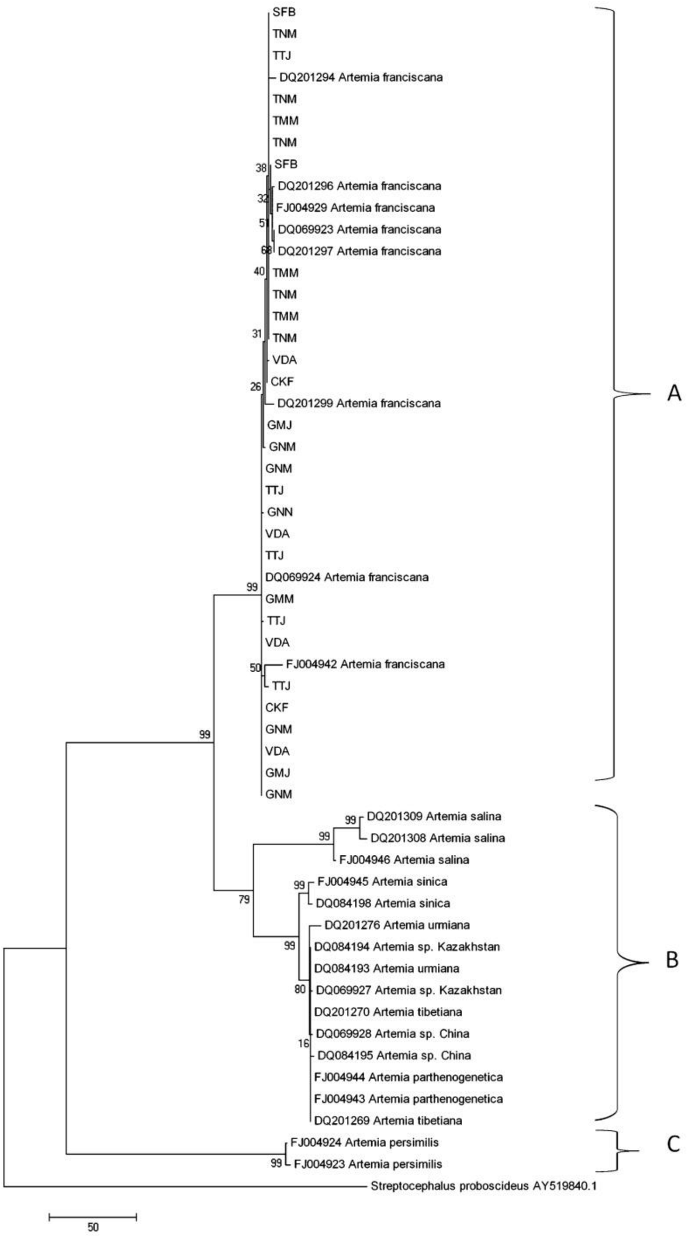


Fig. 3 Principal Coordinate Analysis (PCoA) of the different strains of *Artemia* species. PCoA was performed using the software PAST (Palaeontological Statistics) (Hammer et al., 2001)

Fig. 4 Phylogenetic tree constructed by the maximum parsimony analysis using the Internal Transcribes Spacer 1 (ITS-1) regions of the 29 different Indian adapted *Artemia* and the 26 reference strains obtained from the NCBI with 1,000 bootstrapping. *CKF* Kelambakkam, Tamil Nadu; *VDA* Vedaranyam, Tamil Nadu; *TTJ* Tuticorin, Tamil Nadu; *GMJ* Mithapur, Gujarat; *GNM* Nanganvadi, Gujarat; *TNM*: Thamaraikkulam, Tamil Nadu; *TMM* Marakkanam, Tamil Nadu; *SFB Artemia franciscana* San Francisco Bay strain



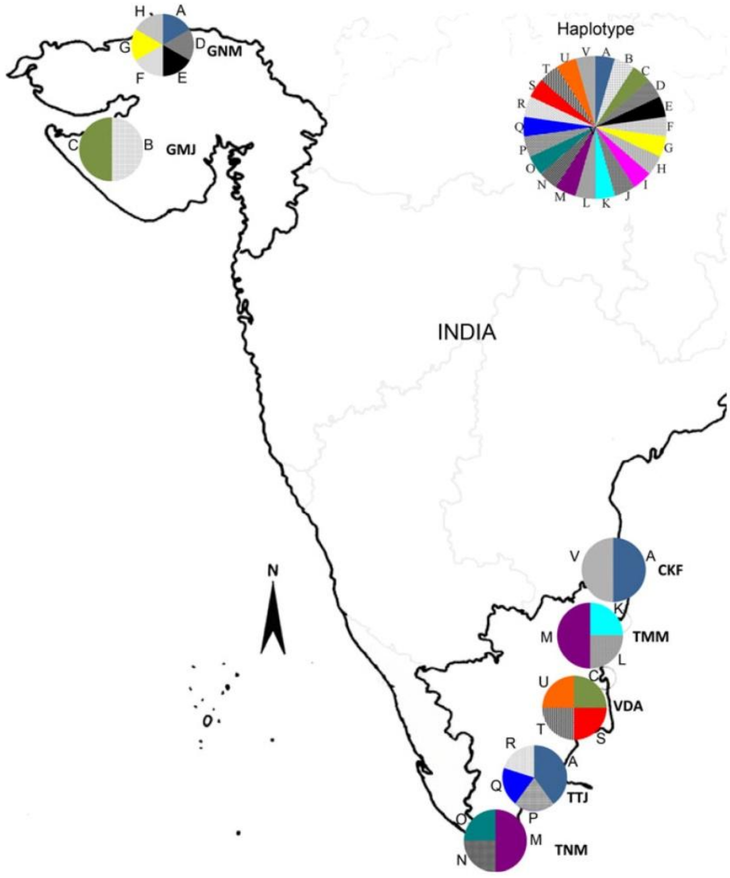


Fig. 5 Haplotype map of the different *Artemia* strains collected from various hypersaline habitats of India. Twenty-two haplotypes (A to V) were assigned individual *colors/hatches. CKF* Kelambakkam, Tamil Nadu; *VDA* Vedaranyam, Tamil Nadu; *TTJ* Tuticorin, Tamil Nadu; *GMJ* Mithapur, Gujarat; *GNM* Nanganvadi, Gujarat; *TNM* Thamaraikkulam, Tamil Nadu; *TMM* Marakkanam, Tamil Nadu

subcontinent. Similarly, haplotype C was distributed in the most distant geographical locations, Southeast region (VDA) and Northwest (GMJ) regions, whereas the haplotype B was restricted to Southeast (TMM and TNM) region of the country. Of the 16 polymorphic nucleotide sites, the parsimony informative sites were found to be few (5) in comparison with singleton variable sites (11). Nucleotide diversity and the population mutation parameter for ITS 1 region were less in the Indian *Artemia* populations (Pi: 0.00283). The overall transition/transversion ratio of the ITS-1 sequences was $R = 0.81$. Tajima's D, based on the number of segregating sites (s) with the nucleotide diversity (π), was -0.96775 ($P < 0.01$).

Discussion

Although parthenogenetic *Artemia* populations have been reported from various geographical regions of India since 1950s, later studies revealed the gradual disappearance of *Artemia* populations from these regions. Parthenogentic *Artemia* populations were reported for both Sambhar and Didwana basins from the northwest region of India (Baid, 1968; Bhargava & Alam, 1980). Alam (1980) reported the disappearance of *Artemia* fauna of Sambhar during 1977–1978. Though Jakher et al. (1990) observed *Artemia* populations in Didwana during 1984–1985; studies by Jain & Marian (2005) during 2000–2004 reported the loss of *Artemia* fauna from the habitat, but failed to ascribe any reasons for its disappearance. The present study reveals that all the *Artemia* populations collected during 2008–2009 were bisexual, composed of fertile males and females, indicating the absence of parthenogenetic *Artemia* populations. Furthermore, the assessment of the reproductive pattern of the *Artemia* samples clearly showed that none of the individually reared, mature forms could produce any offspring confirming the total absence of parthenogenesis. On the contrary, the breeding experiments clearly showed that all these populations were able to crossbreed, producing fully fertile offspring, thus exhibiting their mating efficiency and fertility. Absence of any infertile pairs among the different crosses indicated the absence of any reproductive barrier among the Indian populations and the alien *A. franciscana* (Sugumar, 2010). The variations exhibited in the reproductive output and cyst/nauplii numbers are typical and the entire F2 progeny matured, confirming their fertility.

It should be noted that the non decapsulated cyst diameters of the present Indian populations did not show any significant difference within the samples except for TNM and VDA population. The present Indian *Artemia* sp. cyst size were closer to the exotic *A. franciscana* strains than to the earlier reported native *A. parthenogentica* (John, 1994). In a similar study, widespread distribution of *A. franciscana* in the Western Mediterranean region was reported by Amat et al. (2007) based on adult *Artemia* biometry.

Mean pairwise GD within the Indian *Artemia* populations was found to be negligible (0 to 0.01), indicating that all the Indian populations are genetically similar and can be considered as a single species. The absence of any significant difference in the GD (0 to 0.01) between the different Indian *Artemia* populations and *A. franciscana* indicate their conspecific nature. Nucleotide diversity (Nei & Tajima, 1981) is one of the most commonly used indices for estimating genetic diversity of a population when DNA sequence data for individuals are available. Considerably low nucleotide diversity observed in the Indian *Artemia* populations (Pi: 0.00283) during the present study indicates low genetic diversity. Species level molecular analysis has provided valuable insights into the species composition of the Indian *Artemia* populations. Comparison of the ITS-1 sequence lengths of the various *Artemia* species showed that the Indian populations are very close to that of the exotic *A. franciscana* with 99% homology, while other *Artemia* populations such as *A. urmiana*, *A. tibetiana*, *A. salina*, and *A. persimilis* showed considerable variations. Phylogenetic analysis using the MP and NJ tree methods (Fig. 4) clearly distinguished *A. franciscana* and the Indian *Artemia* populations (clade A) from *A. persimilis* group (clade C). Clade B consisted of two groups, a unique *A. salina* group and a mixed group consisting of *A. sinica, A. urmiana, A. kazakhastan, A. tibetiana, A. china*, and *A. parthenogenetica.* Within this mixed group, our observations indicate a close relationship between *A. parthenogenetica, A. tibetiana, A. china* strain, *A. Kazakhstan*, and *A. urmiana* strain which in agreement with the previous reports postulating that the diploid parthenogentic *Artemia* lineages are closely related to *A. urmiana* (Baxevanis et al., 2006) and *A. Kazakhstan* is closely related to *A. parthenogenetica* group (Muñoz et al., 2008). This was further evidenced from the PCoA, where the *A.urmiana, A. tibetiana, A. china* strain, *A. Kazakhstan*, and *A. parthenogenetica* formed a single cluster (cluster C), distinct from the reference *A. franciscana* and the Indian *Artemia* populations (cluster D), while *A. salina* (cluster A), *A. sinica* (cluster B), and *A. persimilis* (cluster E) appeared as separate clusters (Fig. 3).

Present investigation reports the lack of regional endemism in different *Artemia* populations from geographically distant habitats of the Indian subcontinent indicating that these populations are comparatively young and are in the process of genetic divergence. Kappas et al. (2004) have observed that the peculiar biotic nature of the *Artemia* habitats may lead to the development of endemic groups of *Artemia* in later stages of evolution. Haplotype diversity observed during the present study throws light into the distribution pattern of the Indian *Artemia* populations. The distributions of similar haplotypes in geographically distinct habitats indicate a common origin but are evolving in parallel lines (Parkin & Butlin, 2004). High singleton variation in the ITS 1 sequence as revealed by the sequence characterization is a key indicator of the population expansion while the presence of few parsimony informative sites indicate the relative absence of ‘bottle necks’ in the expansion of this population. The negative Tajima’s *D* higher haplotype diversity, higher singleton variations, and less number of parsimony informative sites in the ITS-1 sequence of the Indian *Artemia* populations signify an excess of low frequency polymorphisms, which again indicate that the population is under expansion and/or positive selection (Tajima, 1989). The transitions to transversion (TI: TV ratio) results for the aligned nucleotide sequences showed a higher frequency of transversion than transitions ($R = 0.81$), which leads to the high frequent transition bias (DeSalle et al., 1987).

The morphological and biometric observations, crossbreeding experiments, molecular analysis data, ITS-1 sequence lengths and high per cent of homology, mean pairwise GD data and phylogenetic analysis clearly proves that the *Artemia* populations collected from the various geographical regions of India are *A. franciscana* adapted during the course of time. These observations indicate that the earlier reported parthenogenetic *Artemia* (Kulkarni, 1953; Baid, 1968; Royan et al., 1970; Achari, 1971; Royan, 1979; Bhargava & Alam, 1980; Basil et al., 1987; Kulasekarapandian et al., 1992; John, 1994) have been displaced by the invaded and naturalized, bisexual, *A. franciscana.* In a similar study, colonization induced extinction of local populations by *A. franciscana* has been reported from Mediterranean habitats (Amat et al., 2005, 2007).

Artemia franciscana, have been exported worldwide since the 1950s for use as live feed in hatcheries. The rapid growth of the Indian aquaculture industry since the early eighties paved the way for the invasion of *A. franciscana* into the Indian ecosystems as a live feed. Occurrence of species or populations in non-native environments reveals their ability to invade and coexist with native populations and in course of time adapt in the new habitat. High degree of phenotypic plasticity helps the invasive species to invade and expand in new habitats in the face of hostile environmental conditions (Browne & Wanigasekera, 2000; Clegg et al., 2000; Tanguay et al., 2004). Generally, sexual populations have high dispersal capacities and a competitive advantage over the asexual species, which leads to invasion and establishment (Wilson, 1992). Amat et al. (1991) views *A. franciscana* as an invasive species around the world as it competes with the native brine shrimp populations due to their superior adaptive capabilities. Further, various physical agents such as wind and water; biological carriers' viz., migratory aquatic birds and predators, etc., likely have contributed to the invasion of this exotic species across the different saline habitats of India.

The disappearance of parthenogentic *Artemia* populations from the northwest region of India could be ascribed to the extreme drought conditions in these habitats (Rao, 2009). In the east coast of the Indian subcontinent, the states of Andhra Pradesh, Orissa, and West Bengal have numerous aquaculture farms and hatcheries where *Artemia* has been extensively used as live feed. But surprisingly, our study revealed total absence of *Artemia* in all the hypersaline water bodies of the region. This could be due to the climatic conditions of the region where these hypersaline habitats are prone to frequent cyclones associated heavy rainfall and floods annually (Khatua & Dash, 2000), preventing the establishment of *Artemia* sp. The North West (Gujarat) and South East (Tamil Nadu) regions have the most suitable climatic conditions and most of the hypersaline areas are naturally protected from flooding, helping the populations to tide over any adverse conditions and flourishes. Numerous hatcheries situated in these areas must have way for the invasion of *A. franciscana* populations into the nearby hypersaline habitats. Moreover, these locations occur in the migratory route of flamingoes and this may also have helped in the dispersal of the species which is in agreement with the views expressed by Green et al. (2005).

Anthropogenic activities have aided the massive invasion of *A. franciscana* into the natural hypersaline habitats in the Indian subcontinent where it has flourished and displaced the native parthenogenetic *Artemia* from its natural habitat. The species–ecosystem and habitat interaction has to be further explored to shed light on this aspect. This forms the first report on the loss of the native parthenogenetic *Artemia* from the Indian hypersaline water bodies following invasion of *A. franciscana*, a species already known for invasions worldwide. Further studies are required to assess the changes in the flora and fauna brought about by such invasions in the concerned regions.

Conclusion

The present investigation sheds light on the massive invasion of alien *Artemia franciscana* species into the Indian saline ecosystems, displacing the native parthenogenetic *Artemia*. The exceptional phenotypic plasticity and broad tolerance to diverse environments and the breeding pattern of the alien *A. franciscana* species facilitated its invasion into the Indian subcontinent. This forms the first report on the displacement of parthenogenetic *Artemia* by the invading *A. franciscana.* Further studies are required to understand the changes brought by such invasions in the particular ecosystems.

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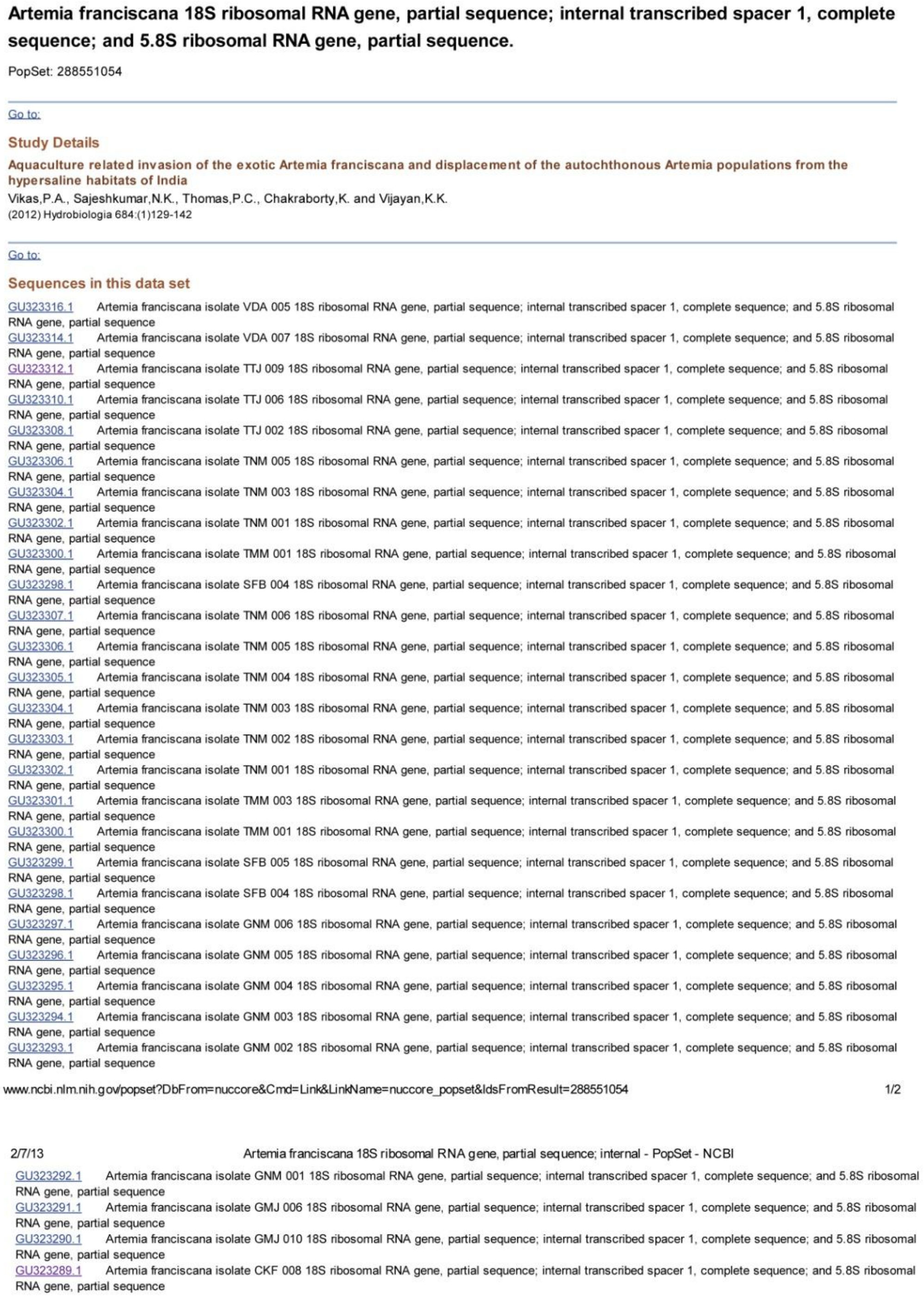

Artemia franciscana 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence.

PopSet: 288551054

Go to:

Study Details

Aquaculture related invasion of the exotic Artemia franciscana and displacement of the autochthonous Artemia populations from the hypersaline habitats of India

Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C., Chakraborty,K. and Vijayan,K.K.

(2012) Hydrobiologia 684:(1)129-142

Go to:

Sequences in this data set

GU323316.1 Artemia franciscana isolate VDA 005 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence

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GU323310.1 Artemia franciscana isolate TTJ 006 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence

GU323308.1 Artemia franciscana isolate TTJ 002 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence

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GU323304.1 Artemia franciscana isolate TNM 003 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence

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2/7/13 Artemia franciscana 18S ribosomal RNA gene, partial sequence; internal - PopSet - NCBI

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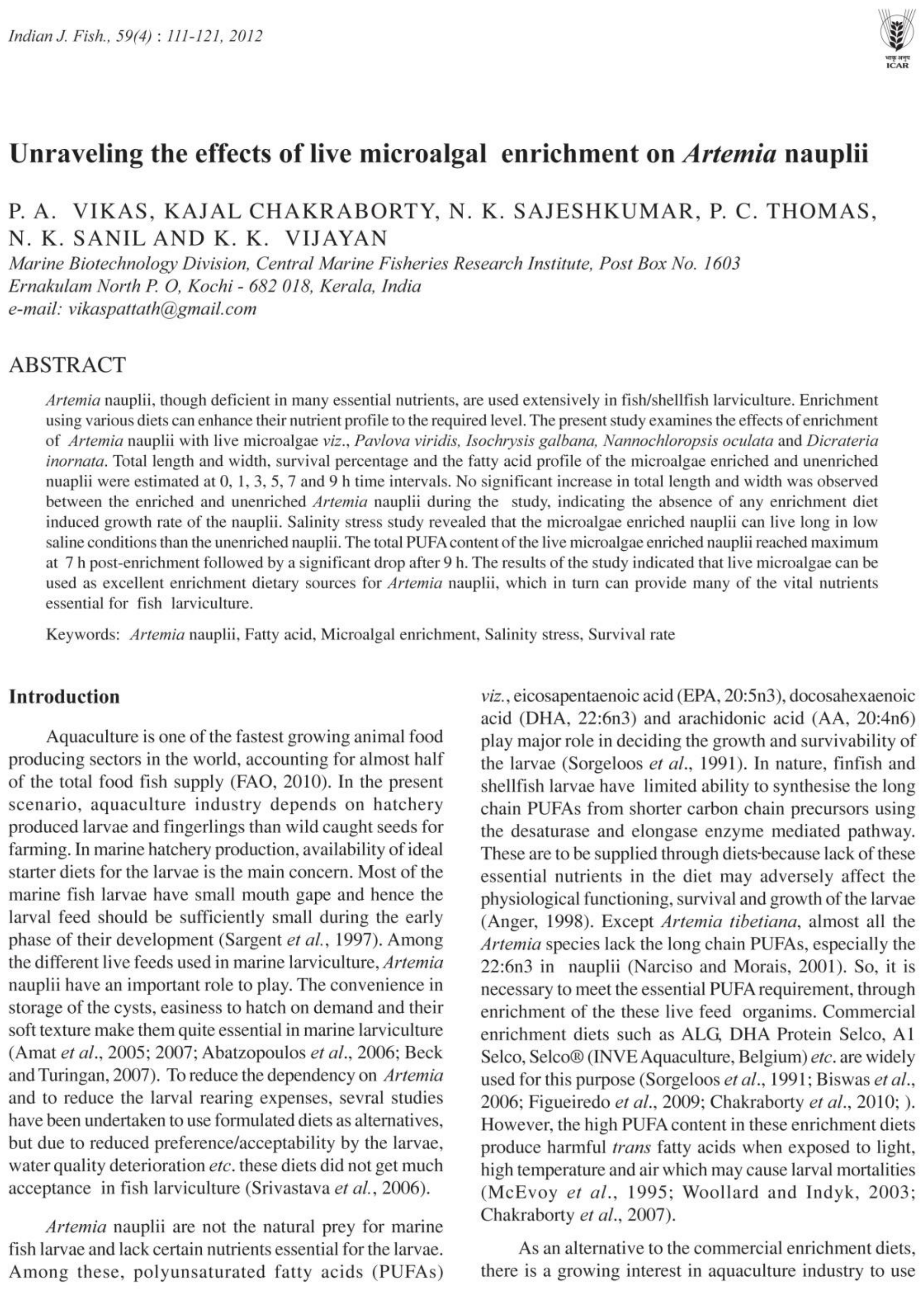

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Unraveling the effects of live microalgal enrichment on *Artemia* nauplii

P. A. VIKAS, KAJAL CHAKRABORTY, N. K. SAJESHKUMAR, P. C. THOMAS, N. K. SANIL AND K. K. VIJAYAN
Marine Biotechnology Division, Central Marine Fisheries Research Institute, Post Box No. 1603
Ernakulam North P. O, Kochi - 682 018, Kerala, India
e-mail: vikaspattath@gmail.com

ABSTRACT

Artemia nauplii, though deficient in many essential nutrients, are used extensively in fish/shellfish larviculture. Enrichment using various diets can enhance their nutrient profile to the required level. The present study examines the effects of enrichment of *Artemia* nauplii with live microalgae *viz*., *Pavlova viridis, Isochrysis galbana, Nannochloropsis oculata* and *Dicrateria inornata*. Total length and width, survival percentage and the fatty acid profile of the microalgae enriched and unenriched nuaplii were estimated at 0, 1, 3, 5, 7 and 9 h time intervals. No significant increase in total length and width was observed between the enriched and unenriched *Artemia* nauplii during the study, indicating the absence of any enrichment diet induced growth rate of the nauplii. Salinity stress study revealed that the microalgae enriched nauplii can live long in low saline conditions than the unenriched nauplii. The total PUFA content of the live microalgae enriched nauplii reached maximum at 7 h post-enrichment followed by a significant drop after 9 h. The results of the study indicated that live microalgae can be used as excellent enrichment dietary sources for *Artemia* nauplii, which in turn can provide many of the vital nutrients essential for fish larviculture.

Keywords: *Artemia* nauplii, Fatty acid, Microalgal enrichment, Salinity stress, Survival rate

Introduction

Aquaculture is one of the fastest growing animal food producing sectors in the world, accounting for almost half of the total food fish supply (FAO, 2010). In the present scenario, aquaculture industry depends on hatchery produced larvae and fingerlings than wild caught seeds for farming. In marine hatchery production, availability of ideal starter diets for the larvae is the main concern. Most of the marine fish larvae have small mouth gape and hence the larval feed should be sufficiently small during the early phase of their development (Sargent *et al.*, 1997). Among the different live feeds used in marine larviculture, *Artemia* nauplii have an important role to play. The convenience in storage of the cysts, easiness to hatch on demand and their soft texture make them quite essential in marine larviculture (Amat *et al*., 2005; 2007; Abatzopoulos *et al*., 2006; Beck and Turingan, 2007). To reduce the dependency on *Artemia* and to reduce the larval rearing expenses, sevral studies have been undertaken to use formulated diets as alternatives, but due to reduced preference/acceptability by the larvae, water quality deterioration *etc*. these diets did not get much acceptance in fish larviculture (Srivastava *et al.*, 2006).

Artemia nauplii are not the natural prey for marine fish larvae and lack certain nutrients essential for the larvae. Among these, polyunsaturated fatty acids (PUFAs) *viz.*, eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3) and arachidonic acid (AA, 20:4n6) play major role in deciding the growth and survivability of the larvae (Sorgeloos *et al*., 1991). In nature, finfish and shellfish larvae have limited ability to synthesise the long chain PUFAs from shorter carbon chain precursors using the desaturase and elongase enzyme mediated pathway. These are to be supplied through diets because lack of these essential nutrients in the diet may adversely affect the physiological functioning, survival and growth of the larvae (Anger, 1998). Except *Artemia tibetiana*, almost all the *Artemia* species lack the long chain PUFAs, especially the 22:6n3 in nauplii (Narciso and Morais, 2001). So, it is necessary to meet the essential PUFA requirement, through enrichment of the these live feed organims. Commercial enrichment diets such as ALG, DHA Protein Selco, A1 Selco, Selco® (INVE Aquaculture, Belgium) *etc*. are widely used for this purpose (Sorgeloos *et al*., 1991; Biswas *et al*., 2006; Figueiredo *et al*., 2009; Chakraborty *et al*., 2010;). However, the high PUFA content in these enrichment diets produce harmful *trans* fatty acids when exposed to light, high temperature and air which may cause larval mortalities (McEvoy *et al*., 1995; Woollard and Indyk, 2003; Chakraborty *et al*., 2007).

As an alternative to the commercial enrichment diets, there is a growing interest in aquaculture industry to use marine microalgae as enrichment diet for the live feeds (Chakraborty *et al*., 2007). Microalgae make up the basis of food chain and serve as the renewable reservoir of PUFAs in nature (Ferreira *et al*., 2008). Studies on the fatty acid composition of many marine microalgae revealed that PUFAs present in them are much more stable than many commercially available enrichment diets (Volkman *et al*., 1989). The 22:6n3: 20:5n3: 20:4n6 ratios in live microalgal cells closely resemble natural larval diets and further, PUFAs in microalgae are better protected against oxidation by the natural antioxidants present in them. Earlier studies on microalgae revealed the importance of *Nannochloropsis, Chaetoceros* and *Chlorella* as suitable enrichment diets for live feeds (Vazhappilly and Chen, 1998). The nutritional value of microalgae can vary significantly depending on the species and their culture conditions. They are amenable to mass culture and biomass scale-up through photobioreactor and/or fermentation technology, and the nutrient profile of these organisms can be optimised to a great extent by manipulating the culture conditions (Martinez-Fernandez, 2006).

The major problems associated with *Artemia* nauplii enrichment are the incidence of naupliar mortality and rapid growth during enrichment. Though the enrichment process can increase the nutrient content of the *Artemia* nauplii, larger size prevents them from being ingested by the small mouthed fish larvae (Sorgeloos *et al*., 2001).

Studies on the viability of nauplii following enrichment in different media are scanty. Incidence of higher naupliar mortality before being ingested by the fish larvae remains another issue since dead nauplii are seldom preferred by fish larvae. According to Sastry (1983), larval survival rate is usually high under optimal environmental conditions, and gets reduced and finally stops when these conditions are not conducive. Since the optimum environmental conditions required for higher survival varies with the species of fish larvae, an enriched live feed which is able to withstand a wide range of environmental conditions will perform better. Most of the enrichment studies on *Artemia* nauplii evaluated the diet induced changes in the enriched nauplii, its nutrient content and growth rate (Narciso, 2000; Han *et al*., 2001; Ritar *et al*., 2004; Figueiredo *et al.*, 2009). Investigations on salinity stress tolerance in microalgae enriched *Artemia* nauplii can unravel the role of exogenous diets in regulating the salinity induced mortality. However, studies to evaluate the salinity stress tolerance of *Artemia* nauplii after enrichment with microalgae are scanty.

The present study was aimed to evaluate the effect of the microalgae, *Pavlova viridis, Isochrysis galbana, Nannochloropsis oculata* and *Dicrateria inornata* on the survival percentage, nauplii length and width as well as fatty acid content of *Artemia* nauplii at different time intervals following enrichment and also to test the salinity stress tolerance of the microalgae enriched nauplii..

Materials and methods

Preparation of microalgal culture for enrichment

Microalgal cultures of *P. viridis, I. galbana, N. oculata* and *D. inornata* were obtained from the marine microalgal culture facility of Central Marine Fisheries Research Institute (CMFRI), India. Microalgae were cultured in Walne's medium (Walne, 1970) in Haffkine glass flasks (3 l) under circadian light: dark cycle (12 h: 12 h) with a light intensity of 2000 lx at a temperature of 24 ± 1 °C. Microalgae were inoculated from a stock culture (7×10^6 cells ml^{-1}) and grown up to stationary phase and were further mass cultured at similar culture conditions. Microalgal cell density was estimated using a Neubauer haemocytometer under a microscope (Leica, Wetzlar, Germany). The cultures were maintained for one week before being used as enrichment diet for *Artemia* nauplii and further biochemical analysis.

Artemia cyst hatching

Allochthonous *Artemia franciscana* cysts obtained from the Indian *Artemia* Reference facility of the Central Marine Fisheries Research Institute, India were used for the study. *A. franciscana* cysts (1 g l^{-1}) were incubated (24 h) in cylindrical culture flasks (5 l) holding autoclaved seawater (35‰ and 27 ± 1 °C) with strong bottom aeration and optimum light (2000 lx) for hatching (Sorgeloos, 1986). Freshly hatched *Artemia* nauplii were harvested and stocked in glass beakers (5 l) and the density was estimated from the subsamples (10 replicates of 1 ml subsamples).

Enrichment experiment

Enrichment experiment was carried out using the four species of microalgae *viz*., *P. viridis, I. galbana, N. oculata* and *D. inornata* as diet. *Artemia* metanuaplii were stocked (@50 nauplii ml^{-1}) in 15 cylindrical enrichment tanks (5 l), each having one of the microalgal diet *i.e*., *P. viridis, I. galbana, N. oculata* or *D. inornata* (in triplicates) at a density of 45±5 X 10^4 cells ml^{-1}along with control group without microalgal diet, in triplicate . Optimum temperature (23 ± 1°C) and strong bottom aeration was maintained in all the nauplii enrichment tanks.

Evaluation of survival rates of the enriched nauplii

Enrichment induced naupliar mortality were computed in all the enrichment tanks (microalgae and control group) at five different time intervals (0, 1, 3, 5, 7 and 9 h). Nauplii survival rates were computed following the subsampling method. The number of live and dead nauplii in 1 ml subsamples (20 times) in all the tanks was counted and the percentage survival was computed [Survival percentage = Number of live nauplii / (Number of live nauplii + Number of dead nauplii)*100]. Thereafter the live nauplii were restocked in the respective enrichment tanks.

Evaluation of nauplii length and width

The total length and width of the microalgae enriched (*P. viridis, I. galbana, N. oculata* and *D. inornata)* and unenriched nauplii (control groups) at 0, 1, 3, 5, 7 and 9 h were measured. In short, *Artemia* nauplii were sampled (200 individuals) from the top, middle and bottom of the enrichment containers at different time intervals and fixed in Lugol's iodine to arrest further growth. Total length (top of the head to the end of the caudal furca) and width of the nauplii were measured under a sterozoom microscope (Leica, Wetzlar, Germany) attached with digital camera (Leica, DFC 290) and image analysis software.

Evaluation of salinity stress tolerance of the enriched nauplii

Salinity stress tolerance of the *Artemia* nauplii were estimated at 9 h post-enrichment.. For this, water having salinity of 0, 5, 10, 20 and 30 ppt was prepared by adding double distilled freshwater to aged seawater (35 ppt). Microalgae enriched nauplii were transferred to cylindrical culture flasks (15 ± 4 nauplii ml^{-1}) containing autoclaved water of salinities 0, 5, 10, 20, 30 and 35 ppt. The nauplii cultures were maintained with continuous aeration at optimum temperature (23 ± 1°C) and light (2000 lx). Survival rates of the enriched nauplii at different time intervals (3, 6 and 12 h) were estimated by counting the live and dead nauplii from 1 ml subsamples (twenty replicates) and the mortality percentage was computed [Mortality % = (Total number of dead individuals/Total live individuals + Total dead individuals)*100]. The results were compared with that of unenriched nauplii.

Estimation of fatty acid content

Fatty acid content of the microalgal enrichment diets (*P. viridis, I. galbana, N. oculata* and *D. inornata*) as well as that of enriched and unenriched nauplii at different time intervals was estimated. The four microalgal enrichment diets used for the experiments were harvested by centrifugation (10,000 rpm for 5 min at 4 °C) at the middle of stationary phase and stored at -80 °C until used for analysis prior to the experiment.

Microalgae enriched and unenriched nauplii samples (1.5 to 1.8 g in triplicate) from each treatment tanks were harvested at 0, 1,3,5,7 and 9 h time intervals during the experiment and rinsed with freshwater. The harvested nauplii were homogenised and the total lipid was extracted following Bligh and Dyer (1959). Fatty acid composition of all the samples were determined as per Metcalf *et al*. (1966). Triglycerides were extracted using $CHCl_3$/MeOH/H_2O (2:4:1, v/v/v), and saponified with alkaline reagent (3 ml, 0.5 N KOH/MeOH). The saponified materials were allowed to react with a methylating mixture (14% BF_3/CH_3OH) to *Trans*-esterify the saponificable material yielding fatty acid methyl esters (FAME) that was later extracted with n-hexane/H_2O (1:2, v/v). After the removal of the aqueous layer, the *n*-hexane layer was passed through Na_2SO_4, concentrated in vacuum, reconstituted in petroleum ether (40-60 °C) and stored at -20 °C until analysis. The esterified fatty acid content of the samples was analysed by gas liquid chromatography (GLC) with FID detector using fatty acid methyl ester standard (Supelco FAME 37 standard).

The data were subjected to analysis of variance (ANOVA) and the means of all parameters were examined for significance ($p<0.05$) using the Duncan multiple range tests using SPSS programme 13.0 (SPSS Inc, Chicago, USA).

Results

Survival percentage of Artemia nauplii during enrichment

Survival rate of *Artemia* nauplii during the enrichment process showed variations at 3, 5, 7 and 9 h enrichments while no mortality was observed after the first one hour of enrichment. The overall mortality rate at 9 h post-enrichment was significantly high ($p<0.05$) in the unenriched nauplii group (5.2 ± 1.8%) when compared to the microalgae enriched nauplii (0.5 ± 1.5 to 3.2 ± 2.2%). Among the different microalgal diets, lowest mortality percentage was observed in *N. oculata* and *D. inornata* diets (0.5 ± 1.5 and 0.6 ± 1.8% respectively) while *P. viridis* enriched nauplii showed the highest mortality percentage (3.2 ± 2.2 %) (Table 1).

Table 1. Mortality percentage of *Artemia* nauplii in different enrichment diets at different time intervals

Time (h)	3	5	7	9
Control	2.3 ± 4.6^{ab}	4.1 ± 4.3^{a}	4.6 ± 1.6^{a}	5.2 ± 1.8^{a}
I. galbana	0.6 ± 1.8^{b}	0.6 ± 1.8^{b}	0.6 ± 1.8^{b}	1.6 ± 2.2^{b}
P. viridis	1.4 ± 3.0^{ab}	1.9 ± 1.4^{ab}	2.2 ± 1.0^{ab}	3.2 ± 2.2^{ab}
N. oculata	0.0^{b}	0.5 ± 1.5^{b}	0.5 ± 1.5^{b}	0.5 ± 1.5^{b}
D. inornata	0.0^{b}	0.6 ± 1.8^{b}	0.6 ± 1.8^{b}	0.6 ± 1.8^{b}

Values are represented as mean ± SD

Column-wise values with different superscripts indicate significant difference ($p<0.05$)

Effect of enrichment time on the total length and width of the nauplii

No significant difference in total length (TL) was observed between the microalgae (*P. viridis, I. galbana, N. oculata* and *D. inornata*) enriched and unenriched nauplii at different post-enrichment time intervals. Total length (TL) of the *Artemia* nauplii in all the experiments increased significantly ($p<0.05$) after 1st hour of enrichment (560.4 ± 75.6 to 582.1 ± 69.5 µm) (Table 2) . *I. galbana* enriched group had the smallest nauplii (600.2 ± 72.0 µm) when compared to the other microalgae enriched nauplii (628.3 ± 50.7 to 614.9 ± 47.3 µm) at 9 h post-enrichment.

Total width of the nauplii significantly reduced ($p<0.05$) at 9 h in all microalgae enriched groups as well as control group (142.5 ± 17.0 to 156.0 ± 20.1 µm) as compared to 0 h (164.6 ± 18.4 µm) (Table 3). No significant difference in nauplii width was apparent between the various microalgae enriched nauplii and unenriched nauplii at different time intervals.

Salinity stress tolerance of the enriched nauplii

No significant mortality percentage was observed after 3 and 6 h of incubation in 0, 5, 10, 20 and 35 ppt salinities in microalgae enriched and unenriched *Artemia* nauplii. At 12 h post-enrichment, nauplii mortality percentage significantly ($p<0.05$) increased in unenriched *Artemia* nauplii (21.1%) when compared to the microalgae enriched nauplii (6.22 to 11.76%) (Table 4). *P. viridis* enriched *Artemia* nauplii showed high salinity stress tolerance compared to the all other microalgae enriched nauplii (Table 4).

Effect of enrichment time on the fatty acid profile of the nauplii

Fatty acid profile of the microalgal diets showed notable differences in the levels of major fatty acids. Saturated fatty acids (SFAs) were found to be low in the microalgae *D. inornata* and *I. galbana* (23.81 and 28.95%, respectively) as compared to *P. viridis* and *N. oculata* (35.16 and 38.64 %, respectively) diets (Table 5a, 5b, 5c and Fig. 1). Impact of enrichment time and microalgal species on the fatty acid profile of the *Artemia* nauplii are illustrated in Table 5a and 5b. Except in *N. oculata* enriched nauplii, a gradual decline in SFAs was obvious in all the microalgae enriched nauplii up to 7 h post-enrichment. After 9th h of enrichment, the SFA content of the nauplii showed a significant ($p<0.05$) increase. Among the different SFAs, fatty acid 14:0 and 16:0 contributed the major share of the total SFA in the nauplii. Interestingly, the 9 h enriched nauplii showed significant positive correlation between the microalgal SFA and enriched nauplii SFA, while no correlation was observed in others. In the unenriched *Artemia* nauplii a gradual increase in total SFA was apparent with time (Table 5c).

The monounsaturated fatty acids (MUFAs) showed a significant decline in all the enriched nauplii when compared to the unenriched nauplii (42.4%). No correlation was observed between the MUFA content in the microalgal

Table 2. Total length (µm) of the *Artemia* nauplii in different enrichment diets at different time intervals

Time (h)	Control	*I. galbana*	*P. viridis*	*N. oculata*	*D. inornata*
0	516.6 ± 61.4^{a}	516.6 ± 61.4^{a}	516.6 ± 61.4^{a}	516.6 ± 61.4^{a}	516.6 ± 61.4^{a}
1	576.6 ± 76.9^{b}	560.4 ± 75.6^{a}	578.4 ± 96.3^{b}	582.1 ± 69.5^{b}	560.0 ± 68.4^{b}
3	593.2 ± 83.8^{b}	575.2 ± 94.6^{b}	609.0 ± 86.2^{bc}	600.0 ± 65.5^{b}	602.5 ± 59.3^{c}
5	614.3 ± 55.3^{b}	593.7 ± 71.8^{b}	612.2 ± 54.7^{bc}	604 ± 45.5^{b}	604.3 ± 40.0^{c}
7	617.7 ± 43.5^{b}	599.2 ± 48.7^{b}	617.7 ± 37.4^{bc}	606.9 ± 44.0^{b}	612.8 ± 40.3^{c}
9	618.6 ± 58.6^{b}	600.2 ± 72.0^{b}	628.3 ± 50.7^{c}	614.9 ± 47.3^{b}	623.1 ± 55.1^{c}

Values are represented as mean ± SD (n = 200)
Column-wise values with different superscripts indicate significant difference ($p<0.05$)

Table 3. Total width (µm) of the *Artemia* nauplii in different enrichment diets at different time intervals

Time (h)	Control	*I. galbana*	*P. viridis*	*N. oculata*	*D. inornata*
0	164.6 ± 18.4^{a}	164.6 ± 18.4^{a}	164.6 ± 18.4^{a}	164.6 ± 18.4^{a}	164.6 ± 18.4^{a}
1	176.8 ± 11.8^{b}	165.3 ± 22.1^{a}	158.3 ± 20.7^{a}	164.0 ± 16.9^{a}	159.6 ± 19.5^{a}
3	157.4 ± 17.5^{a}	157.8 ± 15.8^{a}	156.9 ± 14.0^{a}	153.9 ± 20.3^{ab}	165.8 ± 18.3^{a}
5	153.9 ± 17.3^{a}	155.2 ± 9.07^{a}	156.9 ± 13.3^{a}	147.0 ± 9.75^{b}	161.9 ± 19.1^{a}
7	162.1 ± 17.3^{ac}	161.0 ± 24.6^{a}	163.4 ± 14.9^{a}	151.2 ± 13.7^{ab}	153.9 ± 12.9^{a}
9	143.5 ± 12.7^{c}	151.9 ± 28.1^{a}	156.0 ± 20.1^{a}	142.5 ± 17.0^{b}	151.8 ± 23.0^{a}

Values are represented as mean ± SD (n = 200)
Column-wise values with different superscripts indicate significant difference ($p<0.05$)

Table 5a. Percentage fatty acid composition of microalgae (*P. viridis* and *D. inornata*) *vis-à-vis Artemia* nauplii enriched with microalgae for up to 9 h post-enrichment.

Fatty acids		*Artemia* nauplii enriched with *P. viridis*							*Artemia* nauplii enriched with *D. inornata*					
	P. viridis	0h	1h	3h	5h	7h	9 h	*D. inornata*	0h	1h	3h	5h	7h	9 h
Saturated fatty acids (SFAs)														
12:0	0.29	0.08	0.06	0.05	0.03	0.03	1.38	0.93	0.08	0.06	0.06	0.05	0.07	0.88
13:0	0.04	0.03	ND[a]	ND	ND	ND	0.27	0.62	0.03	0.03	0.01	ND	ND	0.25
14:0	11.25	3.53	3.49	3.38	2.74	2.65	5.09	1.35	3.53	3.26	2.59	3.15	3.8	6.92
15:0	0.86	1.39	1.34	1.22	0.41	0.38	0.85	0.59	1.39	1.28	0.57	0.52	0.48	1.24
16:0	19.37	19.3	19.2	18.7	18.3	17.6	22.5	18.91	19.3	19.1	18.6	18.2	18.2	23.4
17:0	0.14	1.52	1.48	1.35	1.28	1.19	1.53	0.10	1.52	1.38	1.29	1.25	0.51	0.84
18:0	1.92	5.1	5.04	4.85	4.24	4.18	7.27	0.48	5.1	5.29	5.64	5.87	4.55	5.06
20:0	0.11	0.04	ND	ND	ND	ND	0.08	0.08	0.04	0.29	0.55	0.73	0.56	0.82
22:0	0.03	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.08
24:0	1.15	0.12	0.09	0.06	0.05	0.03	0.15	0.75	0.12	0.64	1.07	1.38	0.98	1.21
ΣSFA	35.16	31.1	30.7	29.7	27.1	26	39.1	23.81	31.1	31.3	30.4	31.1	29.2	40.7
Monounsaturated fatty acids (MUFAs)														
14:1n9	0.38	0.03	ND	ND	0.03	0.05	0.06	0.23	0.03	0.03	ND	ND	ND	ND
16:1n7	27.9	13.6	13.5	12	12	10.1	9.32	0.64	13.6	12.6	11.5	8.82	7.5	7.19
16:1n9	0.52	0.06	0.04	ND	ND	ND	ND	8.16	0.06	0.05	0.03	0.03	0.03	0.03
18:1n7	1.64	0.12	0.06	0.03	0.03	ND	ND	0.13	0.12	0.1	0.08	0.06	0.05	0.05
18:1n9	7.41	28.5	28.4	27.5	26.3	24.6	21.5	23.57	28.5	27.7	25.1	24.1	22.5	22.1
24:0	0.86	0.08	0.05	0.03	ND	ND	ND	0.34	0.08	0.06	0.05	0.03	0.03	0.12
ΣMUFA	38.71	42.4	42.1	39.6	38.3	34.8	30.9	33.07	42.4	40.6	36.8	33.1	30.2	29.4
Polyunsaturated fatty acids (PUFAs)														
18:2n6 cis	3.48	7.52	8.2	8.61	8.74	8.92	8.82	6.3	7.52	9.83	10.4	10.6	10.8	8.56
18:3n6	1.05	1.18	1.29	1.67	2.24	2.68	0.53	0.39	1.18	1.35	1.42	1.54	1.68	1.19
18:4n6	0.72	0.03	0.09	0.16	0.22	0.62	0.46	0.14	0.03	ND	ND	ND	ND	ND
18:3n3	1.47	3.21	3.82	3.91	4.19	4.26	3.09	12.84	3.21	3.53	5.92	5.89	6.72	5.26
18:4n3	2.9	0.08	0.35	0.49	0.58	0.87	0.73	8.65	0.08	0.2	1.17	1.35	2.26	1.93
C20:2n6	0.24	0.15	0.19	0.21	0.16	0.25	0.18	0.19	0.15	0.23	0.45	0.57	0.65	0.41
C20:3n6	0.18	1.1	1.23	1.34	1.48	1.70	1.15	1.06	1.1	1.35	1.37	1.51	1.93	1.28
C20:4n6	1.86	1.35	1.14	1.62	2.07	2.31	1.82	0.98	1.35	1.42	1.61	1.73	1.93	0.85
20:5n3	9.54	3.08	3.26	3.79	4.15	7.58	4.12	6.82	3.08	3.12	3.19	4.18	5.98	3.06
22:5n3	0.16	0.02	0.04	0.06	0.09	0.48	0.32	0.38	0.02	ND	0.03	0.12	0.08	0.06
22:6n3	1.81	0.32	0.43	1.48	2.72	1.37	0.85	1.26	0.32	0.36	1.09	1.95	1.85	0.82
ΣPUFA	23.41	18	20	23.3	26.6	31	22.1	39.01	18	21.4	26.6	29.4	33.9	23.4
Σ n3	15.88	6.71	7.9	9.73	11.7	14.6	9.11	29.95	6.71	7.21	11.4	13.5	16.9	11.1
Σ n6	7.53	11.3	12.1	13.6	14.9	16.5	13	9.06	11.3	14.2	15.2	15.9	17	12.3
n3/n6	2.11	0.59	0.65	0.71	0.79	0.88	0.7	3.31	0.59	0.51	0.75	0.85	0.99	0.91
Σ PUFA/Σ SFA	0.67	0.58	0.65	0.79	0.98	1.19	0.56	1.64	0.58	0.68	0.88	0.94	1.16	0.58
DHA/EPA	0.19	0.10	0.13	0.39	0.65	0.18	0.21	0.18	0.10	0.12	0.34	0.47	0.31	0.27

The individual fatty acid is expressed as the percentage of total fatty acids.

[a] ND, fatty acid identified on GC trace but not integrated by the instrument.

ΣSFA : total SFAs; ΣMUFA; total MUFAs and ΣPUFA: total PUFAs

diet and the enriched nauplii. Total MUFA of the enriched nauplii is mainly contributed by 18:1n9 (60%) followed by 16:1n 7 (30%). Compared to SFA, MUFA content was low in all the enriched nauplii throughout the enrichment period except in *I. galbana* enriched nauplii at 9 h post-enrichment (41.7%). Among the microalgae sources evaluated, *I. galbana* (43.31%) showed the highest PUFA content followed by *D. inornata* (39.01%), *P. viridis* (23.41%) and *N. oculata* (19.81%). Variation in the total PUFA was clear in the microalgae enriched nauplii at different durations of enrichment (Table 5a and b). Total PUFA in the nauplii increased slightly up to 7 h post-enrichment in *I. galbana, P. viridis* and *D. inornata* diets (29.3, 31 and 33. 9% respectively) and at 9 h post-enrichment the PUFA was reduced considerably to 18.5, 22.1 and 23.4% respectively. But, the total PUFA of the *N. oculata* enriched nauplii reached a maximum at 5 h post-enrichment (27.6 %) and later showed a decline after the 7 (25.7%) and 9 h post-enrichment (12.9 %) (Table 5a and 5b).

Among the different PUFAs observed in the enriched nauplii, 18:2n6 cis, C20:4n6 and 20:5n3 contributed the major share irrespective of the specific microalgal diet used. 18:3n3 and 20:5n3 was high at 7th h of enrichment with *D. inornata* (6.72% and 5.98%), *I. galbana* (4.54 and 4.11%) and *P. viridis* (4.26 and 7.58%) while in *N. oculata* enriched nauplii, it reached the maximum level (3.69 and 7.81%) at 5th h of enrichment. However, 20:5n3 was high (7.81%) at 5 h post-enrichment in *N. oculata* enriched nauplii and was 7.58% in *P. viridis* enriched nauplii at 7th h post-enrichment. *N. oculata* enriched nauplii showed an incremental trend in 20:4n6 and 18:3n6 content up to the 5th h of enrichment (2.15 and 1.56% TFA, respectively),

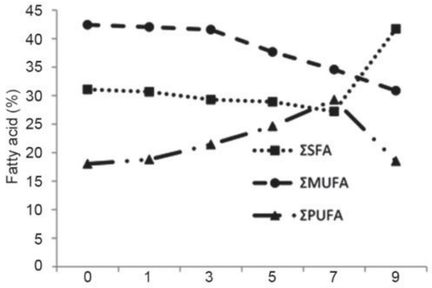
Table 5b. Percentage fatty acid composition of microalgae (*N. oculata* and *I. galbana*) *vis-à-vis Artemia* nauplii enriched with microalgae for up to 9 h post-enrichment

Fatty acids		*Artemia* nauplii enriched with *N. oculaa*							*Artemia* nauplii enriched with *I. galbana*					
	N. oculata	0h	1h	3h	5h	7h	9 h	*I. galbana*	0h	1h	3h	5h	7h	9 h
Saturated fatty acids (SFAs)														
12:0	0.94	0.08	0.06	0.05	0.05	0.03	2.6	0.56	0.08	0.03	ND	ND	ND	0.13
13:0	0.29	0.03	ND	ND	ND	ND	0.12	0.08	0.03	ND	ND	ND	ND	0.05
14:0	5.19	3.53	3.32	3.2	3.15	3.2	5.14	6.35	3.53	3.41	3.34	3.35	2.68	5.14
15:0	1.07	1.39	1.33	1.2	1.18	1.73	2.16	0.81	1.39	1.36	1.27	1.23	0.91	1.73
16:0	21.59	19.3	19.2	19.1	18.8	20.6	27.2	14.5	19.3	19.2	18.3	18.1	17.8	26.2
17:0	0.09	1.52	1.48	1.48	1.42	1.36	3.94	0.03	1.52	1.5	1.48	1.47	1.25	1.86
18:0	8.76	5.1	4.47	4.51	4.43	3.65	9.84	5.93	5.1	5.06	4.89	4.74	4.5	6.38
20:0	0.11	0.04	0.05	0.03	ND	ND	0.63	0.05	0.04	ND	ND	ND	0.03	0.08
22:0	0.06	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24:0	0.54	0.12	0.1	0.08	0.06	0.05	0.17	0.64	0.12	0.08	0.05	0.05	0.06	0.18
ΣSFA	38.64	31.1	30	29.7	29.1	30.7	51.8	28.95	31.1	30.7	29.3	28.9	27.2	41.7
Monounsaturated fatty acids (MUFAs)														
14:1n9	0.08	0.03	0.03	0.02	ND	ND	0.03	0.19	0.03	ND	ND	ND	0.05	ND
16:1n7	16.88	13.6	13.2	12.7	12	12	8.3	3.59	13.6	13.5	13.2	11.5	10.9	9.24
16:1n9	0.13	0.06	0.04	0.03	ND	ND	ND	0.15	0.06	0.04	0.04	0.03	ND	ND
18:1n7	0.14	0.12	0.09	0.06	0.04	0.04	0.11	0.08	0.12	0.08	0.05	0.05	0.03	0.03
18:1n9	19.5	28.5	27.9	27.3	25.1	24.3	21.2	20.25	28.5	28.3	28.2	26.1	23.6	21.6
17:01	0.05	ND	ND	0.08	0.05	0.03	0.07	ND	ND	ND	ND	ND	ND	ND
24:00	1.23	0.08	0.06	0.04	0.09	0.06	0.19	0.28	0.08	0.06	0.08	0.05	0.03	0.03
ΣMUFA	38.01	42.4	41.3	40.2	37.3	36.4	29.9	24.54	42.4	42	41.6	37.7	34.6	30.9
Polyunsaturated fatty acids (PUFAs)														
18:2n6 cis	4.63	7.52	7.69	8.13	8.75	8.83	4.15	8.48	7.52	8.14	9.09	10	10.6	6.27
18:3n6	0.35	1.18	1.23	1.38	1.56	1.43	0.64	0.15	1.18	1.15	1.21	1.39	1.45	0.51
18:4n6	0.04	0.03	ND	ND	0.05	0.02	ND	0.04	0.03	0	0.15	0.18	0.64	0.47
18:3n3	0.67	3.21	3.38	3.42	3.69	3.5	1.92	5.78	3.21	3.25	3.56	4.18	4.54	4.05
18:4n3	0.15	0.08	0.15	0.18	0.23	0.14	0.09	15.32	0.08	0.13	0.21	0.26	0.34	0.28
C20:2n6	0.82	0.15	0.21	0.29	0.36	0.25	0.18	0.08	0.15	0.18	0.21	0.2	0.39	0.25
C20:3n6	0.54	1.1	1.24	1.72	1.76	1.72	0.18	0.19	1.1	0.98	1.15	1.24	1.32	0.86
C20:4n6	2.15	1.35	1.42	1.86	2.15	1.72	1.51	0.48	1.35	1.42	1.59	1.68	2.21	1.39
20:5n3	9.69	3.08	4.87	5.09	7.81	6.93	3.63	2.60	3.08	3.27	3.58	4.05	4.11	3.03
22:5n3	0.13	0.02	ND	0.06	0.06	0.03	ND	0.44	0.02	ND	ND	0.03	0.05	ND
22:6n3	0.64	0.32	0.12	0.11	1.22	1.15	0.62	9.75	0.32	0.28	0.69	1.38	3.69	1.41
ΣPUFA	19.81	18	20.3	22.2	27.6	25.7	12.9	43.31	18	18.8	21.4	24.6	29.3	18.5
Σ n3	11.28	6.71	8.52	8.86	13	11.8	6.26	33.89	6.71	6.93	8.04	9.9	12.7	8.77
Σ n6	8.53	11.3	11.8	13.4	14.6	14	6.66	9.42	11.3	11.9	13.4	14.7	16.6	9.75
n3/n6	1.32	0.59	0.72	0.66	0.89	0.84	0.94	3.60	0.59	0.58	0.6	0.67	0.77	0.90
Σ PUFA/Σ SFA	0.51	0.58	0.68	0.75	0.95	0.84	0.25	1.50	0.58	0.61	0.73	0.85	1.08	0.44
DHA/EPA	0.07	0.1	0.02	0.02	0.16	0.17	0.17	3.75	0.1	0.09	0.19	0.34	0.9	0.47

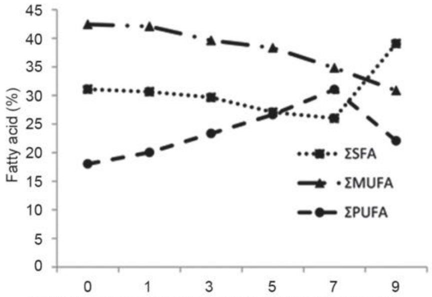
while in all others it reached maximum after 7th h of enrichment. Total n3 PUFAs were high in *N. oculata* enriched *Artemia* nauplii which ranged from 8.52 (at 1 h post-enrichment) to 13% (at 5 h post-enrichment) (Fig. 1). Total PUFA in control gradually decreased with time and lowest percentage was observed at 9 h post-enrichment (Table 5c).

Table 5c. Percentage fatty acid composition of unenriched *Artemia* nauplii at different time intervals

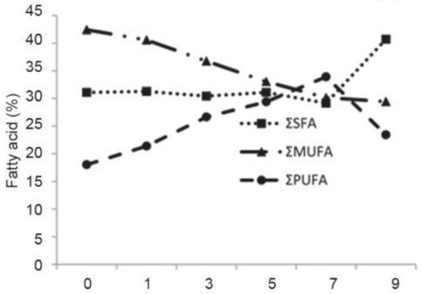
Fatty acids	Incubation time (h)					
	0	1	3	5	7	9
Saturated fatty acids (SFAs)						
12:0	0.08	0.06	0.06	0.09	0.07	1.01
13:0	0.03	ND	ND	ND	ND	0.27
14:0	3.53	4.5	4.7	5.2	6.99	7.87
15:0	1.39	1.4	1.5	1.7	1.87	1.41
16:0	19.3	22	24.5	26.8	27.9	28.8
17:0	1.52	1.8	1.78	2.1	1.89	1.6
18:0	5.1	6	5.99	6.44	7.87	8.2
20:0	0.04	ND	ND	ND	ND	0.08
24:0	0.12	0.09	0.09	0.09	0.08	0.15
ΣSFA	31.11	35.85	38.62	42.42	46.67	49.39
Monounsaturated fatty acids (MUFAs)						
14:1n9	0.03	0.02	0.08	0.03	0.05	0.06
16:1n7	13.6	12.1	11	10.8	9.8	8.8
16:1n9	0.06	0.07	0.02	0.04	0.03	0.02
18:1n7	0.12	0.21	0.03	0.19	0.16	0.13
18:1n9	28.5	27.6	25.6	23.4	21.5	19.8
24:0	0.08	0.1	0.03	0.01	0.07	0.05
ΣMUFA	42.39	40.1	36.76	34.47	31.61	28.86
Polyunsaturated fatty acids (PUFAs)						
18:2n6 cis	7.52	7.5	7.3	6.89	6.01	4.38
18:3n6	1.18	1.29	1.67	1.54	1.14	0.99
18:4n6	0.03	0.09	0.16	0.22	0.62	0.02
18:3n3	3.21	3.11	2.98	2.55	2.01	1.98
18:4n3	0.08	0.12	0.14	0.1	0.2	0.34
C20:2n6	0.15	0.13	0.26	0.16	0.25	0.18
C20:3n6	1.1	1.23	1.01	0.98	0.88	1.15
C20:4n6	1.35	1.14	1.15	0.93	0.67	1.87
20:5n3	3.08	2.98	2.88	2.12	1.98	1.6
22:5n3	0.02	0.04	0.06	0.09	0.04	0.05
22:6n3	0.32	0.28	0.2	0.19	0.11	0.1
ΣPUFA	18.04	17.91	17.81	15.77	13.91	12.66
Σ n3	6.71	6.53	6.26	5.05	4.34	4.07
Σ n6	11.33	11.38	11.55	10.72	9.57	8.59
n3/n6	0.59	0.57	0.54	0.47	0.45	0.47
Σ PUFA/Σ SFA	0.58	0.50	0.46	0.37	0.30	0.26
DHA/EPA	0.10	0.09	0.07	0.09	0.06	0.06



I. galbana enrched *Artemia* at different time intervals (h)



P. viridis enrched *Artemia* at different time intervals (h)



D. inornata enrched *Artemia* at different time intervals (h)

Fig. 1. The differential composition of ΣPUFA (total polyunsaturated fatty acid), ΣSFA (total monounsaturated fatty acid) and, ΣMUFA (total monounsaturated fatty acids) in *Artemia* nauplii enriched with microalgae at different time intervals

Discussion

Prey size has always been a key limiting factor influencing the feeding efficiency of the predatory larvae. Barros and Valenti (2003) suggested the most favorable relationship of prey size to predator length as 0.2, indicating the importance of small size prey in larviculture. In the present study, no significant increase in total length and width was apparent between the microalgae enriched and

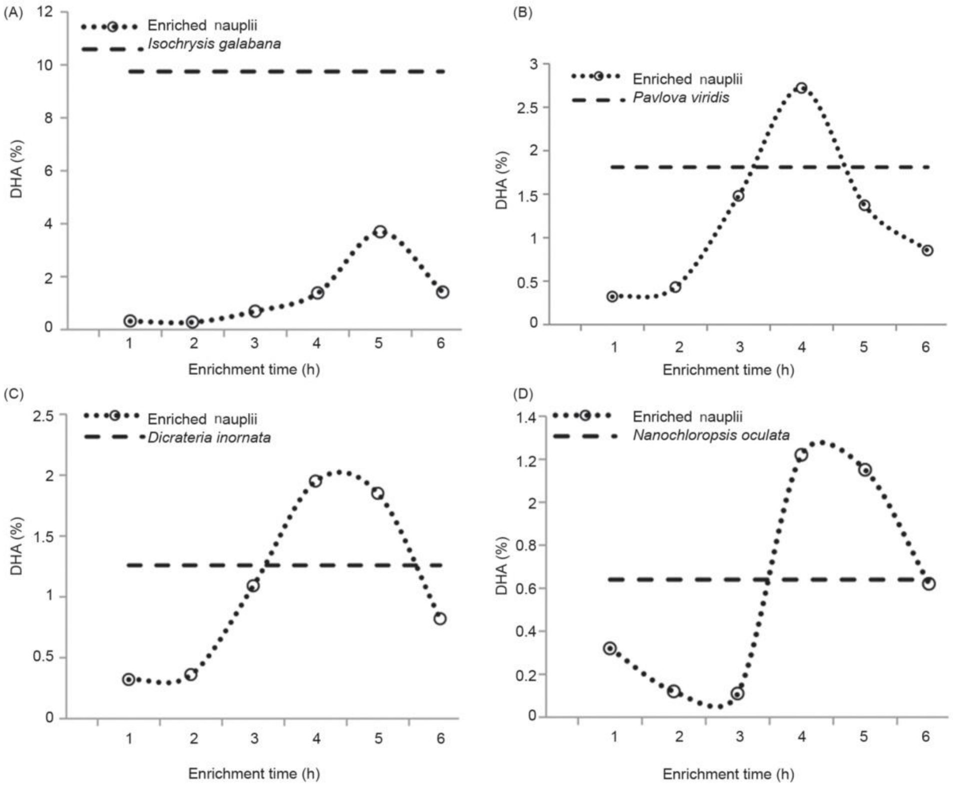


Fig. 2. 22:6n 3 content of the microalgal diets and the enriched *Artemia* nauplii

unenriched *Artemia* nauplii during the 9 h enrichment study, indicating the absence of any enrichment diet induced overgrowth in length and width of the nauplii.

One of the major drawbacks associated with the enrichment of *Artemia* nauplii using different diets is the reduced naupliar survival rates. Low mortality rate observed in the microalgae enriched nauplii when compared to the unenriched nauplii could be attributed to the presence of biomolecules such as betaine, inosine 5-monophosphate and free amino acids in the microalgal diet (Muller-feuga, 2000; Kolkovski *et al*., 1997). Figueiredo *et al*. (2009) while studying the enrichment of *Artemia* nauplii with Algamac 2000, observed that naupliar mortality increased with enrichment time and temperature and suggested that it could be due to elevated bacterial load and enhanced metabolism at higher temperatures.

Major environmental parameters which limit the development and survival of larvae in aquaculture include salinity, temperature, pH, *etc*., their specific requirement varying with the species reared (Paul and Paul, 1999). Among these, except in the case of euryhaline species, salinity stands out as the most important factor affecting the culture conditions. Superiority of a live feed in larviculture is primarily based on their versatility to acclimatise to sudden environmental changes when added to the larval rearing tanks.

The present observations show that nauplii enriched with microalgae have higher tolerance to variations in salinity especially at 0 ppt, indicating their better osmotic stress tolerance when compared to the unenriched nauplii. Fish larvae always prefer to ingest moving or swimming nauplii and the percentage of dead nauplii in larval culture tanks will result in reduced feeding and lead to water quality problems and disease outbreaks. Thus tolerance to varying salinity conditions is always an advantage while using nauplii as live feed in larval rearing. The very long-chain fatty acids (VLCFA) assimilated from the microalgae may have played a role in protecting the membrane integrity thereby raising the salinity tolerance while facing the osmotic changes associated with the various salinity regimes. Prasitchoke *et al*. (2007) observed that VLCFAs are important for membrane construction and stabilisation in eukaryotic cells. Though the commercial enrichment diets are also rich in VLCFAs, it has been observed earlier that the nauplii enriched with the commercial enrichment diets are already weakened/stressed which might have further reduced their salinity tolerance. The high salinity stress tolerance of the microalgae enriched nauplii can improve the feeding efficiency and the water quality conditions in the larval rearing systems, especially in low saline conditions.

Fatty acids, especially the polyunsaturated fatty acids (PUFAs) have a vital role in deciding the survival, growth and development of finfish larvae. The long chain PUFAs *viz.,* 20:5n3 and 20:4n6 are mostly involved in the production and modulation of eicosanoids (Brown, 1994), while the 22:6n3 function to keep the structural and functional integrity in larval cell membranes, including neural function (Bell *et al.,* 2003; Chakraborty *et al*., 2007). However, finfish larvae have a very limited ability to synthesise PUFAs in required quantity to meet their demand. Freshly hatched nauplii of *A. franciscana* showed high 20:5n3 (3.08%) with rather low 22:6n3 (0.32%) and 20:4n6 (1.35%), which is in agreement with the reports by several authors (Han *et al*., 2001; Narciso and Morais, 2001, Kara, *et al*., 2004). This demands the supplementation of essential fatty acids in the nauplii before feeding the larvae (Sargent *et al*., 2002; Bell *et al.,* 2003; Monroig *et al.,* 2006).

Though only small amounts of C_{20} fatty acids were present in *I. galbana* and *D. inornata* , the precursors for the biosynthesis of C_{20} PUFAs namely C_{18} PUFAs 18:2n6 cis and 18:4n3 were present in large amounts. Fatty acid content in the enriched nauplii varied as a function of microalgal dietary treatment and enrichment time. Essential fatty acids *viz.,* 20:5n3 and 22:6n3 increased with the progress of enrichment up to 5-7 h post-enrichment. Among all the microalgal dietary sources studied, *I. galbana* had high 22:6n3, C_{18} fatty acids and total PUFA followed by *D. inornata* and *P. viridis*. Nauplii enriched with *I. galbana* for 7 h had the highest 22:6n3 content (3.69%), followed by *P. viridis* (2.7%) and *D. inornata* (2%) after 5 h of enrichment (Fig. 2). The 22:6n3 content of *Artemia* nauplii during the present study was far higher than that of *Artemia* nauplii enriched with a mixture of *Nannochloropsis salina, Chaetoceros calcitrans* and *Chlorella salina* (Chakraborty *et al*., 2007). Figueiredo *et al*. (2009) reported 6.45% of 22:6n3 in *Artemia* nauplii enriched with ALG which has a DHA content of 27% showing an assimilation of 23.88%. The present study shows that *Artemia* nauplii enriched with live microalgae with an average DHA content of 3.37% has succeeded in assimilating an average 2.01% of 22:6n3, indicating a far higher percentage (59.88%) of 22:6n3 assimilation.

The results of the present study shows that among the microalgae studied, *N. oculata* had the highest 20:5n3 content. *N. oculata* and *I. galbana* enriched nauplii yielded notable 20:5n3 at 5th (7.81%) and 7th h (7.58%) respectively. This was found to be higher than the baker's yeast and microalgae enriched *Artemia salina* nauplii (4.2%) and *Odonus niger* liver oil enriched *A. franciscana* nauplii (2.5 to 5.1%) suggesting their superiority as enrichment diets (Chakraborty *et al*., 2007; Immanuel *et al*., 2007). The drop in 20:5n3 after the 7th h of enrichment may be due to inadequate rate of desaturation (by Δ5-fatty acid desaturase) and elongation of 18:3n3 or 18:4n3 (Chakraborty *et al*., 2007). The higher 20:5n3 (2.6%) and 22:6n3 (9.75%) content observed in *I. galbana* enriched nauplii points out their ability to effectively amass enough PUFAs from the microalgae.

The total PUFA content of the live microalgae enriched nauplii reached maximum by the 7th h of enrichment (except in *N. oculata* enriched nauplii) followed by a significant drop after the 9th h. Except in *D. inornata,* 18:3n"3 contributed a very low percent of the total PUFA in all the microalgae (3.38 and 13.34%) suggesting the nutritional superiority of live microalgae as an enrichment diet. Low linolenic acid (18:3n"3) in enrichment diets can increase the bioconversion of 20:5n3 to 22:6n3 (Buzzi *et al*., 1996). This study has proved that short time enrichment with *N. oculata* (5 h) can improve the PUFA profile of the nauplii while a minimum of 7 h is required for enrichment with *I. galbana, D. inornata* and *P. viridis* .

Selected live microalgae or their combinations can be used as excellent enrichment dietary sources for *Artemia* nauplii, which in turn can provide many of the vital nutrients essential for the fish larvae in larviculture. The present study reveals that 5 to 7 h of enrichment with live microalgal diets can significantly improve the essential PUFA content while keeping the nauplii size at minimum. The high survival rate of the live algae enriched nauplii will enhance feeding of the fish larvae and reduce the deterioration of water quality in rearing tanks. Further, the high salinity stress tolerance observed in the microalgae enriched nauplii makes them suitable live feed for a variety of fish species under different salinity regimes.

Present study explored the suitability of using live microalgae as enrichment diets for *Artemia* nauplii. Microalgae were found to be superior enrichment dietary sources for *Artemia* nauplii due to their higher survival rate, nutritional content and salinity tolerance when compared to the commercial enrichment diets. Further, it was observed that short term enrichment of *Artemia* nauplii with live microalgae can enhance the nutritive value of the nauplii while keeping their size at minimum.

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NCBI National Center for Biotechnology Information

List of Sequences submitted to NCBI

Sl No	Title	GenBank Accession no	Authors
1.	*Artemia franciscana* isolate CKF 008 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323289	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
2.	*Artemia franciscana* isolate CKF 003 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323317	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
3.	*Artemia franciscana* isolate GMJ 010 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323290	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
4.	*Artemia franciscana* isolate GMJ 006 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323291	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
5.	*Artemia franciscana* isolate GNM 001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323292	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
6.	*Artemia franciscana* isolate GNM 002 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323293	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
7.	*Artemia franciscana* isolate GNM 003 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323294	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
8.	*Artemia franciscana* isolate GNM 004 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323295	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
9.	*Artemia franciscana* isolate GNM 005 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323296	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
10.	*Artemia franciscana* isolate GNM 006 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323297	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.

11.	*Artemia franciscana* isolate SFB 004 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323298	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
12.	*Artemia franciscana* isolate SFB 005 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323299	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
13.	*Artemia franciscana* isolate TMM 001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323300	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
14.	*Artemia franciscana* isolate TMM 003 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323301	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
15.	*Artemia franciscana* isolate TNM 001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323302	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
16.	*Artemia franciscana* isolate TNM 002 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323303	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
17.	*Artemia franciscana* isolate TNM 003 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323304	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
18.	*Artemia franciscana* isolate TMM 004 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323305	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
19.	*Artemia franciscana* isolate TNM 005 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323306	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
20.	*Artemia franciscana* isolate TNM 006 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323307	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
21.	*Artemia franciscana* isolate TTJ 002 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323308	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
22.	*Artemia franciscana* isolate TTJ 004 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323309	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
23.	*Artemia franciscana* isolate TTJ 006 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323310	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
24.	*Artemia franciscana* isolate TTJ 008 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323311	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
25.	*Artemia franciscana* isolate TTJ 009 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequenceTTJ 009	GU323312	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.

26.	*Artemia franciscana* isolate VDA 003 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323313	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
27.	*Artemia franciscana* isolate VDA 007 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323314	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
28.	*Artemia franciscana* isolate VDA 010 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323315	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
29.	*Artemia franciscana* isolate VDA 005 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	GU323316	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
30.	*Artemia franciscana* voucher CMFRI:08 CKF 051 small heat shock protein (Hsp22) gene, partial cds	GU377282	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
31.	*Artemia franciscana* voucher CMFRI:08 GMJ 051 small heat shock protein (Hsp22) gene, partial cds	GU377283	Vikas,P.A., Sajeshkumar,N.K., Thomas,P.C. and Vijayan,K.K.
32.	*Artemia* sp. PCT-2008 isolate CKF 001 cytochrome oxidase subunit I(COI) gene, partial cds; mitochondrial.	FJ467923	Thomas,P.C., Vijayan,K.K., Sajeshkumar,N.K., Vikas,P.A. and Paulton,M.P.
33.	Artemia sp. PCT-2008 isolate GMJ 001 cytochrome oxidase subunit I(COI) gene, partial cds; mitochondrial.	FJ467924	Thomas,P.C., Vijayan,K.K., Sajeshkumar,N.K., Vikas,P.A. and Paulton,M.P.
34.	Artemia sp. PCT-2008 isolate VDA 001 cytochrome oxidase subunit I(COI) gene, partial cds; mitochondrial.	FJ467926	Thomas,P.C., Vijayan,K.K., Vikas,P.A., Sajeshkumar,N.K. and Paulton,M.P.
35.	Artemia sp. PCT-2008 isolate SFB 001 cytochrome oxidase subunit I(COI) gene, partial cds; mitochondrial	FJ467925	Thomas,P.C., Vijayan,K.K., Vikas,P.A., Sajeshkumar,N.K. and Paulton,M.P.

World Conference on Marine Biodiversity (WCMB), 26-30 September 2011 at Aberdeen, Scotland (UK)

Analysis of the genetic and biochemical adaptations of Arthropods to withstand salt stress in hypersaline habitats; A case study

Vikas P.A, Sajeshkumar N.K, Kajal Chakraborty, Thomas P.C and Vijayan K.K.

Marine Biotechnology Division, Central Marine Fisheries Research Institute, Indian Council of Agricultural Research, PB. No.1603, Ernakulam North PO, Cochin-18, Kerala, India.
0919447993080, vikaspattath@gmail.com

Identification of differential expression/ composition of genetic and biochemical indicators viz., trehalose, carotenoids, amino acids, soluble proteins and long chain fattyacid elongase genes (LCFAE) is an essential way to find out their functional role in stress resistance. In the present study, we analyzed the said biochemical parameters vis-à-vis the genes (LCFAE gene) encoding the essential PUFAs in the hypersaline candidate Arthropod, *Artemia franciscana*, incubated under different saline regimes (50-200‰ with a difference of 50‰) up to a duration of 24 h. Real time expression of the LCFAE gene revealed that the LCFAE gene acts as a salt stress protector by up regulating the LCFAE gene expression at the higher salinities (150 & 200‰). This catalyzed the elongation of short chain fattyacids to form the long chain fatty acids, viz., 20:5n 3, 22:6n 3 and other 26-carbon precursors required for ceramide and sphingolipid synthesis to protect the cell surface from salt stress through the production of stable and chemically resistant outer leaflet of the plasma membrane lipid bilayer. The study also unraveled the role of soluble protein, essential & non essential amino acids, carotenoid and trehalose in salt stress resistance in candidate Arthropods.

World Conference on Marine Biodiversity (WCMB), 26-30 September 2011 at Aberdeen, Scotland (UK)

ANALYSIS OF THE REAL TIME EXPRESSION OF HEAT SHOCK PROTEIN 22 GENE (Hsp22) AND TREHALOSE ON STRESS RESISTANCE IN THE CRYPTOBIOTIC *ARTEMIA* AND IT'S LIFE STAGES

Vikas P.A*, Sajeshkumar N.K, Kajal Chakraborty, Thomas P.C and Vijayan K.K

Central Marine Fisheries Research Institute (CMFRI)
Marine Biotechnology Division (MBTD)
Cochin-682 018, Kerala, India
vikaspattath@gmail.com

The brine shrimp *Artemia* (Branchiopoda, Anostraca) are widely distributed in salterns and hypersaline water bodies throughout the world and it exhibit extreme stress tolerance. During stress*,* molecular chaperones interact with other proteins to modulate folding, cell localization and functionality, and to protect against irreversible denaturation. Among the different molecular chaperones, small heat shock proteins (sHsp) are important because of their protective capability, representing the first line of defense against physiological and environmental stresses. Trehalose, alpha-linked disaccharide formed by an α, α-1, 1-glucoside bond between two α-glucose units also provided protection to biological materials against dehydration and desiccation, and impart the ability to revive the organisms from the cryptobiotic state. The present study was conducted to analyze the role of small heat shock protein (Hsp) 22 and trehalose in the cryptobiotic Artemia and its life stages.

Artemia samples were collected from the hypersaline habitats of Kelambakam (CKF), Vedaranyam (VDA), Tuticorin (TTJ), Mithapur (GMJ), Tamaraikulam (TNM), and Marakanam (TMM) of India. Transcription levels of hsp22 in the different developmental stages of *Artemia* (cyst, adult and nauplii) were estimated through Real-time PCR (IQ5 Thermal thermal cycler, BioRad, USA). Total RNA of the *Artemia* were isolated and the cDNA was reverse transcribed using the RT reagent kit with oligo d(T)18 as primer.

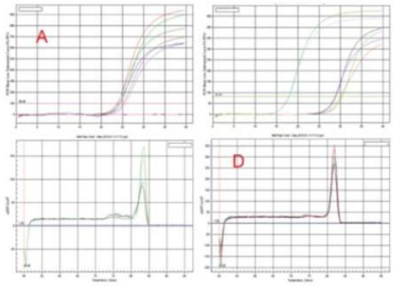


Figure 1. (A & B) Amplification plots of hsp 22 and β actin (C & D) Dissociation curve (-dR/dT vs. T) (R = normalized fluoresence; T = temperature) shows single independent dissociation peaks for *Artemia* cyst, nauplii and adult.

RT PCR amplification of hsp22 from the cDNA was carried out using specific primers. The β-actin used as housekeeping gene. The relative expression ratio of target mRNAs and the CT values revealed exceptionally higher Hsp22 expression in cysts than the nauplii and adult (Fig 2). High molecular chaperon activity of the hsp22 observed in cryptobiotic cyst with diapause-destined embryos enable them to resist the adverse environmental conditions better than the nauplii and adult. This provides an insight into the switch over of hsp22 expression during the favorable conditions. The hsp22 sequenced generated in the present study was deposited in NCBI (GU377282 & GU377283). Trehalose content in the cyst, nauplii and adult of *Artemia* from different indigenous and reference strains were estimated and revealed the higher trehalose holding ability of cyst than nauplii and adult (Fig 2). Hatching percentage of the different *Artemia* cysts were also worked out and correlated with the trehalose content. Pearson correlation analysis revealed positive correlation (0.781) between the hatching percentage and trehalose content (P<0.5, Fig 3). The result is indicative of the protective role of trehalose over the embryos leading to enhanced hatching. It is proposed that trehalose content of the cyst could be a potential biomarker index to assess the hatching quality of the *Artemia* cyst.

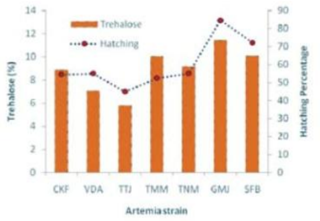


Figure 2. Correlative response of the cyst trehalose and hatching percentage of different *Artemia* strain

IDENTIFICATION AND CHARACTERISATION OF LONG CHAIN FATTY ACID ELONGASE GENE IN THE BRINE SHRIMP *Artemia*

Sajeshkumar N.K, Vikas.P.A, Thomas.P.C, Paulton. M.P and Vijayan.K.K

Marine Biotechnology Division
Central Marine Fisheries Research Institute
Kochi, Kerala, India
sajesh72@gmail.com

Brine shrimp, *Artemia,* are widely distributed in the inland and coastal salterns all over the world. It is the most widely used live feed as it is the easiest live feed to prepare and have high lipid content especially poly unsaturated fatty acids (PUFA) essential for finfish and crustacean larval rearing. Studies on the biosynthetic pathways of PUFA in diverse group of animals have proved the importance of desaturases and elongases in PUFA biosynthesis. Elongase is critical in the biosynthetic pathway involved in the production of long chain polyunsaturated fatty acids. However no information concerning the sequence of elongase gene of crustaceans is available until now. With this background, to understand the biosynthetic network of fatty acids in *Artemia* sp. an attempt was made to identify the genes encoding fatty acid elongase, as part of the efforts to generate the molecular genetic database on genes involved in fatty acid production and expression in *Artemia*.

Synthesis of the cDNA encoding long chain fatty acid elongase from the *Artemia* of Indian origin was carried out by reverse-transcription of RNA isolated from the *Artemia* nauplii following standard methods. PCR amplification of the elongase gene was carried out using degenerate primers which were designed based on the conserved motifs in the elongase sequence of *Oncorhynchus mykiss, Clarias gariepinus, Sparus aurata, Danio rerio, Pediculus humanus corporis, Aedes aegypti and Aedes albopictus*. The forward primer Deg ELO-2F and the reverse primer Deg ELO-1R targeted the conserved regions EFMDTFFF and WWKK respectively. A segment of elongase gene in the cDNA was then amplified through PCR which involved an initial denaturation step at 95^{0}C for 4 min, followed by 50 cycles of denaturation at 95^{0}C for 30 sec, annealing at 58^{0}C for 40 sec and extension at 72^{0}C for 45 sec and a final extension at 72^{0}C for 7 min. The PCR products were sequenced and searched for homology at the Genbank Nucleotide database of NCBI using the tblastn programme. The sequenced showed 72% identity to the PUFA elongase of *P. humanus corporis*, 71% identity to *A. aegypti*, *A.albopictus* and other insects, as both Crustaceans and insects comes under the phylum Arthropoda. Aminoacid sequence was deduced and BLAST searched using protein database at NCBI and the result showed high homology (95%) with long chain fatty acid elongases of several insects.

Here, we report the partial sequence of fatty acid elongase gene in *Artemia* responsible for the elongation of fatty acid to another longer one.The present study shall reveal the full sequence of the fatty acid elongase gene of *Artemia* and this may be the first report of the sequence of elongase gene from the marine Crustaceans. The database generated out of this study can be used for future work; genetic improvement programs through genetic engineering and marker assisted selective breeding for PUFA rich *Artemia* and other Crustaceans.

Table1. BLAST result of the Amino acid sequence of fatty acid elongase gene of *Artemia*

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query covera
BAG30735.1	similar to CG31523-PA [Papilio xuthus]	241	241	95%
XP_397228.2	PREDICTED: similar to CG31523-PA, isoform A [Apis mellifera]	239	239	95%
XP_313358.3	AGAP003600-PA [Anopheles gambiae str. PEST] >gb|EAA08894.3| AG	238	238	95%
XP_002432211.1	elongation of very long chain fatty acids protein, putative [Pediculus hu	238	238	95%
XP_001600017.1	PREDICTED: similar to elongase, putative [Nasonia vitripennis]	237	237	95%
XP_002095954.1	GE25348 [Drosophila yakuba] >gb|EDW95666.1| GE25348 [Drosophil	237	237	95%
NP_730843.2	CG31522, isoform A [Drosophila melanogaster] >gb|AAF52122.3| CG3	236	236	95%

372

MOLECULAR PHYLOGENY OF INDIAN ARTEMIA STRAINS

Sajeshkumar, N. K*., Vikas, P. A., Thomas, P. C., Paulton, M. P. and Vijayan, K. K.

Marine Biotechnology Division
Central Marine Fisheries Research Institute
P.B.No:1603
Ernakulam North P.O, PIN: 682018
Kerala, India
sajesh72@gmail.com

Brine shrimp, Artemia (Crustacea, Anostraca), are widely distributed and reported from more than 600 locations both inland salt lakes and coastal salterns, around the world. The genus Artemia comprises a complex of bisexual species and a large number of parthenogenetic populations under the binomial nomenclature *Artemia parthenogenetica*. Molecular genetic information has been increasingly used to detect the population genetic structure and genetic diversity among morphologically similar populations of the same species.

The occurrence of Artemia was first reported in India from the salt ponds at Vadala, on the outskirts of Bombay. According to earlier reports, the natural populations of Artemia in India was considered to be *Artemia parthenogenetica* and later bisexual forms were also identified and reported from different regions of the country, though a conclusive characterization does not exist. In this study mitochondrial cytochrome oxidase 1 (CO1) gene sequence were used to investigate the geographically separated Artemia populations from different salt works of the country. Based on the DNA sequences available in NCBI database, a specific and sensitive pair of PCR primer was designed using the Primer3 software. The custom synthesized primers were used to amplify the CO1 gene and produce an amplicon of 800bp size.

The amplified gene segments were sequenced and the sequence information was aligned using ClustalX method. Sequences from indigenously collected strains (CKF, TTJ, GMJ, GNM, TNM, TKM, CKM, and TMM), commercial strains (SFB, TBS, and VVC) and from NCBI data base (ASI, ASLI, and APR) were used for phylogenetic analysis. Three species viz. ASI, ASLI, APR were included in the analysis as outgroups. Phylogenetic relationships were analysed using the Neighbour-Joining (NJ) method with bootstrapping (1,000) using MEGA version 4.0

The analysis revealed two groups; one consisting of indigenously collected strains and commercial strains while the other group was consisted of the outgroups. The first group in turn had two clads consisting of both indigenous and commercial strains. The phylogenetic analysis in this study reveals no geographical basis in the distribution of Artemia in India.

Phylogenetic studies with other mitochondrial genes like 16S, Cytochrome b and nuclear gene Elongase are in progress and can reveal more conclusive distribution pattern of the indigenous Artemia strains.

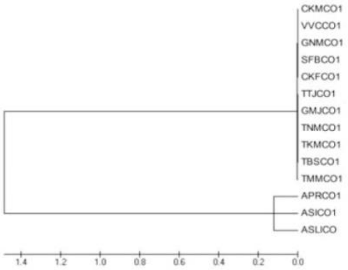
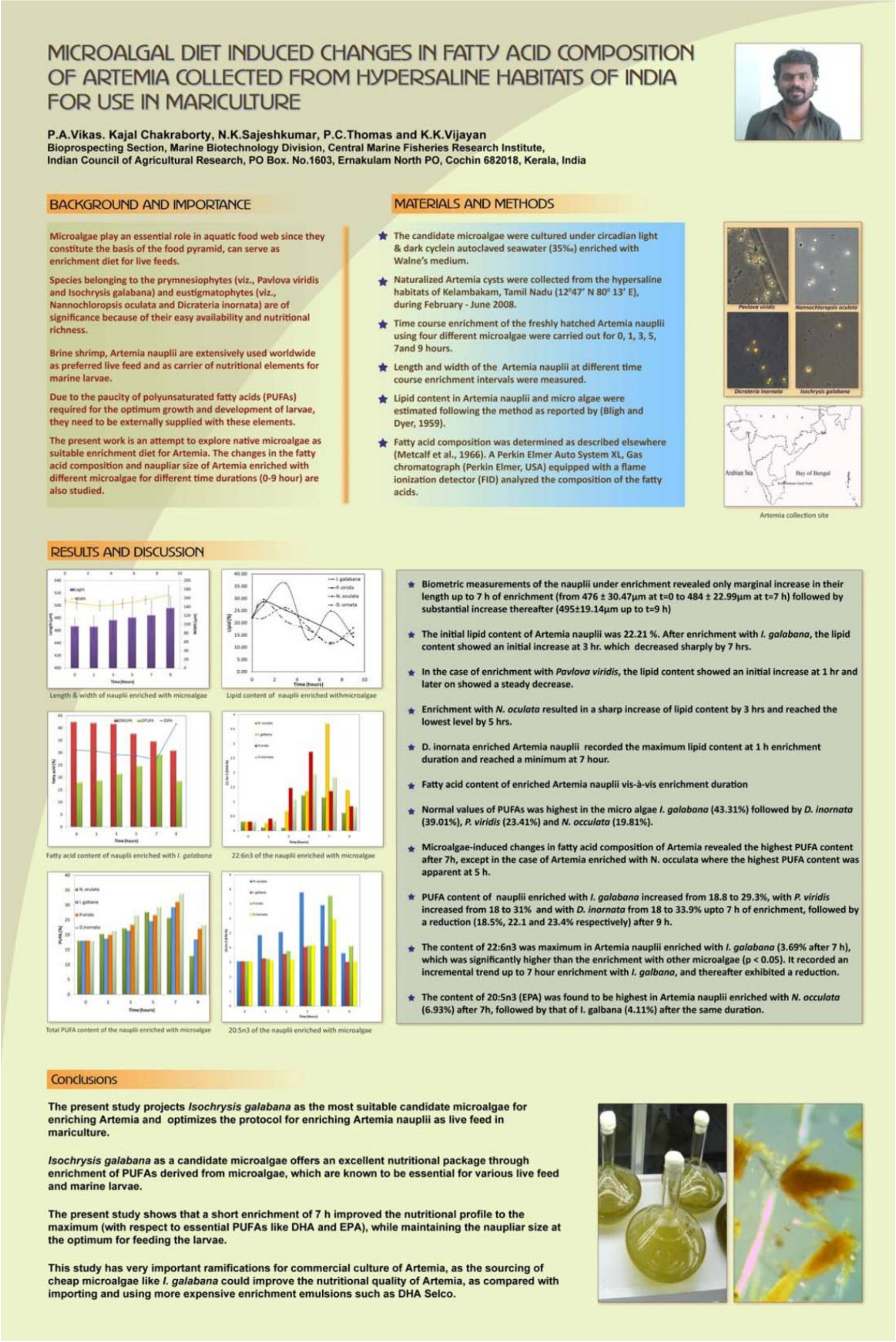


Fig 1. Phylogram consensus tree for Neighbour-Joining analysis inferred from CO1 gene of mitochondrial DNA.



MICROALGAL DIET INDUCED CHANGES IN FATTY ACID COMPOSITION OF ARTEMIA COLLECTED FROM HYPERSALINE HABITATS OF INDIA FOR USE IN MARICULTURE

P.A.Vikas. Kajal Chakraborty, N.K.Sajeshkumar, P.C.Thomas and K.K.Vijayan
Bioprospecting Section, Marine Biotechnology Division, Central Marine Fisheries Research Institute,
Indian Council of Agricultural Research, PO Box. No.1603, Ernakulam North PO, Cochin 682018, Kerala, India

BACKGROUND AND IMPORTANCE

Microalgae play an essential role in aquatic food web since they constitute the basis of the food pyramid, can serve as enrichment diet for live feeds.

Species belonging to the prymnesiophytes (viz., Pavlova viridis and Isochrysis galabana) and eustigmatophytes (viz., Nannochloropsis oculata and Dicrateria inornata) are of significance because of their easy availability and nutritional richness.

Brine shrimp, Artemia nauplii are extensively used worldwide as preferred live feed and as carrier of nutritional elements for marine larvae.

Due to the paucity of polyunsaturated fatty acids (PUFAs) required for the optimum growth and development of larvae, they need to be externally supplied with these elements.

The present work is an attempt to explore native microalgae as suitable enrichment diet for Artemia. The changes in the fatty acid composition and naupliar size of Artemia enriched with different microalgae for different time durations (0-9 hour) are also studied.

MATERIALS AND METHODS

- The candidate microalgae were cultured under circadian light & dark cyclein autoclaved seawater (35‰) enriched with Walne's medium.
- Naturalized Artemia cysts were collected from the hypersaline habitats of Kelambakam, Tamil Nadu (12°47′ N 80° 13′ E), during February - June 2008.
- Time course enrichment of the freshly hatched Artemia nauplii using four different microalgae were carried out for 0, 1, 3, 5, 7and 9 hours.
- Length and width of the Artemia nauplii at different time course enrichment intervals were measured.
- Lipid content in Artemia nauplii and micro algae were estimated following the method as reported by (Bligh and Dyer, 1959).
- Fatty acid composition was determined as described elsewhere (Metcalf et al., 1966). A Perkin Elmer Auto System XL, Gas chromatograph (Perkin Elmer, USA) equipped with a flame ionization detector (FID) analyzed the composition of the fatty acids.

Pavlova viridis
Nannochloropsis oculata
Dicrateria inornata
Isochrysis galabana

Arabian Sea
Bay of Bengal

Artemia collection site

RESULTS AND DISCUSSION

Length & width of nauplii enriched with microalgae

Lipid content of nauplii enriched withmicroalgae

Fatty acid content of nauplii enriched with *I. galabana*

22:6n3 of the nauplii enriched with microalgae

Total PUFA content of the nauplii enriched with microalgae

20:5n3 of the nauplii enriched with microalgae

- Biometric measurements of the nauplii under enrichment revealed only marginal increase in their length up to 7 h of enrichment (from 476 ± 30.47µm at t=0 to 484 ± 22.99µm at t=7 h) followed by substantial increase thereafter (495±19.14µm up to t=9 h)
- The initial lipid content of Artemia nauplii was 22.21 %. After enrichment with *I. galabana*, the lipid content showed an initial increase at 3 hr. which decreased sharply by 7 hrs.
- In the case of enrichment with *Pavlova viridis*, the lipid content showed an initial increase at 1 hr and later on showed a steady decrease.
- Enrichment with *N. oculata* resulted in a sharp increase of lipid content by 3 hrs and reached the lowest level by 5 hrs.
- D. inornata enriched Artemia nauplii recorded the maximum lipid content at 1 h enrichment duration and reached a minimum at 7 hour.
- Fatty acid content of enriched Artemia nauplii vis-à-vis enrichment duration
- Normal values of PUFAs was highest in the micro algae *I. galabana* (43.31%) followed by *D. inornata* (39.01%), *P. viridis* (23.41%) and *N. occulata* (19.81%).
- Microalgae-induced changes in fatty acid composition of Artemia revealed the highest PUFA content after 7h, except in the case of Artemia enriched with N. occulata where the highest PUFA content was apparent at 5 h.
- PUFA content of nauplii enriched with *I. galabana* increased from 18.8 to 29.3%, with *P. viridis* increased from 18 to 31% and with *D. inornata* from 18 to 33.9% upto 7 h of enrichment, followed by a reduction (18.5%, 22.1 and 23.4% respectively) after 9 h.
- The content of 22:6n3 was maximum in Artemia nauplii enriched with *I. galabana* (3.69% after 7 h), which was significantly higher than the enrichment with other microalgae ($p < 0.05$). It recorded an incremental trend up to 7 hour enrichment with *I. galbana*, and thereafter exhibited a reduction.
- The content of 20:5n3 (EPA) was found to be highest in Artemia nauplii enriched with *N. occulata* (6.93%) after 7h, followed by that of I. galbana (4.11%) after the same duration.

Conclusions

The present study projects *Isochrysis galabana* as the most suitable candidate microalgae for enriching Artemia and optimizes the protocol for enriching Artemia nauplii as live feed in mariculture.

Isochrysis galabana as a candidate microalgae offers an excellent nutritional package through enrichment of PUFAs derived from microalgae, which are known to be essential for various live feed and marine larvae.

The present study shows that a short enrichment of 7 h improved the nutritional profile to the maximum (with respect to essential PUFAs like DHA and EPA), while maintaining the naupliar size at the optimum for feeding the larvae.

This study has very important ramifications for commercial culture of Artemia, as the sourcing of cheap microalgae like *I. galabana* could improve the nutritional quality of Artemia, as compared with importing and using more expensive enrichment emulsions such as DHA Selco.

9th Indian Fisheries Forum (9th IFF), 19-23 December, Chennai, India

Extensive use of Alien *Artemia* strains in larviculture industry in India: The Impact on native *Artemia* biotopes

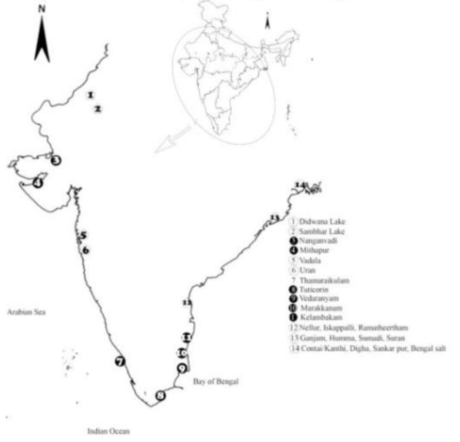
VIKAS P.A*, SAJESHKUMAR N.K, THOMAS P.C, KAJAL CHAKRABORTY, VIJAYAN K.K

Marine Biotechnology Division (MBTD), Central Marine Fisheries Research Institute (CMFRI), Post Box No.1603, Ernakulam North P.O, Cochin-682 018, Kerala, India.

** Mobile: 0091 9447993980; Email: vikaspattath@gmail.com*

In India, *Artemia* has been used as live food in the hatcheries since the early eighties and presently the Indian aquaculture industry consumes approximately 200 tones of *Artemia* annually, which has been met through imports. To study the impacts of exotic *Artemia* strains in the native *Artemia* biotopes, an extensive survey was carried out in the hypersaline habitats, inland salt lakes and salt pans covering seven States and three Union Territories in India. Water and soil samples, cysts and biomass samples were collected to test the occurrence of *Artemia* and identify the species (Fig 1). Morphological observations and molecular analysis using the Internally Transcribed Spacer-1 sequence of the *Artemia* populations collected from Vedaranyam, Tamaraikulam, Marakkanam, Nanganvadi, Mithapur, Kelambakam and Tuticorin regions showed 99% homology with the exotic *Artemia franciscana*. The pairwise genetic distance results of ITS 1 sequences revealed the genetic resemblance of Indian *Artemia* populations with the non-native invasive species *A. franciscana.*

Fig 1. Schematic map showing the survey sites in India



Protein fingerprinting of the whole cell proteins of the *Artemia* strains from Indian salinas vis-à-vis reference *A. franciscana* indicated minor variations with respect to homomeric polypeptide band numbers, molecular weight, and intensity. Nei's genetic identity and genetic distance analyses of the polypeptide bands apparently indicated that the present *Artemia* strains collected from Indian salinas have more similarity with *A. franciscana* than *Artemia salina*. Extensive use of imported *Artemia* cysts paved the way to its spread and establishment in the different hypersaline habitats of India, by various physical and biological agents. Invasion by non-native species is second only to habitat loss as a threat to global biodiversity with a huge economic impact, and has had its greatest impact in aquatic ecosystems. Higher haplotype diversity, singleton variations, and less number of parsimony informative sites in the ITS-1 sequence of the present *Artemia* strains signifies an excess of low frequency polymorphisms thus indicating population size expansion and/or positive selection. Our study conclude that the presently existing *Artemia* populations in Indian salinas are recently invaded *A. franciscana* species and further studies are required to understand the changes brought by expanding populations in the particular ecosystems.

9th Indian Fisheries Forum (9th IFF), 19-23 December, Chennai, India

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Appendix

Aligned Internally Transcribed Spacer-1 sequence of all the Artemia samples

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                   10         20         30         40         50         60         70
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4     GCGTTTGATA AACGAGAAGG AGACTTCTTG GCCATGTGTG GTCAGTAGTT TTTTCCGAAT CGTTGTTATT
SFB C5     .......... .......... .......... .......... .......... .......... ..........
CKF A8     .......... .......... .......... .......... .......... .......... ..........
GMJ 10     .......... .......... .......... .......... .......... .......... ..........
GMJ S6     .......... .-........ .......... .......... .......... .......... ..........
GNM L1     .......... .......... .......... .......... .......... .......... ..........
GMM L2     .......... .......... .......... .......... .......... .......... ..........
GNM L3     .......... .......... .......... .......... .......... .......... ..........
GNM L4     .......... .....A.... .......... .......... .......... .......... ..........
GNN L5     .......... .......... .......... .......... .......... .......... ..........
GNN L6     .......... .......... .......... .......... .......... .......... ..........
TMM G1     .......... .......... .......... .......... .......... .......... ..........
TMM G3     .......... .......... .......... .......... .......... .......... ..........
TNM F1     .......... .......... .......... .......... .......... .......... ..........
TNM F2     .......... .....A.... .......... .......... .......... .......... ..........
TNM F3     .......... .......... .......... .......... .......... .......... ..........
TMM F4     .......... .......... .......... .......... .......... .......... ..........
TNM F5     .......... .......... .......... .......... .......... .......... ..........
TNM F6     .......... .......... .......... .......... .......... ...-...... ..........
TTJ D2     .......... .-........ .......... .......... .......... .......... ..........
TTJ D4     .......... .......... .......... .......... .......... .......... ..........
TTJ D6     .......... .......... .......... .......... .......... .......... ..........
TTJ D8     .......... .......... .......... .......... .......... .......... ..........
TTJ D9     .......... .......... .......... .......... .......... .......... ..........
VDA B3     .......... .......... .......... .......... .......... .....-.... ..........
VDA B7     .......... .-........ .......... .......... .......... .......... ..........
VDA B10    .......... .......... .......... .......... .......... .......... ..........
VDA B5     .......... .......... .......... .......... .......... .......... ..........
CKF A3     .......... .......... .......... .......... .......... .......... ..........

                   80         90        100        110        120        130        140
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4     GACACACGTC CCCGTGTTTG ATGTTTGAGG TGCACAAGTC GGTTTTGCCT ATTGTGAGGG GGTGGTACCC
SFB C5     .......... .......... .......... .......... .......... .......... ..........
CKF A8     .......... .......... .......... .......... .......... .......... ..........
GMJ 10     .......... .......... .......... .......... .......... .......... ..........
GMJ S6     .......... .......... .......... .......... .......... .......... ..........
GNM L1     .......... .......... .......... .......... .......... .......... ..........
GMM L2     .......... .......... .......... .......... .......... .......... ..........
GNM L3     .......... .......... .......... .......... .......... .......... ..........
GNM L4     .......... .......... .......... .......... .......... .......... ..........
GNN L5     .......... .......... .......... .......... .......... .......... ..........
GNN L6     .......... .......... .......... .......... .......... .......... ..........
TMM G1     .......... .......... .......... .......... .......... .......... .C........
TMM G3     .......... .......... .......... .......... .......... .......... ..........
TNM F1     .......... .......... .......... .......... .......... .......... ..........
TNM F2     .......... .......... .......... .......... .......... .......... ..........
TNM F3     .......... .......... .......... .......... .......... .......... ..........
TMM F4     .......... .......... .......... .......... .......... .......... ..........
TNM F5     .......... .......... .......... .......... .......... .......... ..........
TNM F6     .......... .......... .......... .......... .......... .......... ..........
TTJ D2     .......... .......... .......... .......... .......... .......... ..........
TTJ D4     .......... .......... .......... .......... .......... .......... ..........
TTJ D6     .......... .......... .......... .......... .......... .......... ..........
TTJ D8     .......... .......... .......... .......... .......... .......... ..........
TTJ D9     .......... .......... .......... .......... .......... .......... ..........
VDA B3     .......... .......... .......... .......... .......... .......... ..........
VDA B7     .......... .......... .......... .......... .......... .......... ..........
VDA B10    .......... .......... .......... .......... .......... .......... ..........
VDA B5     .......... .......... .......... .......... .......... .......... ..........
CKF A3     .......... .......... .......... .......... .......... .......... ..........
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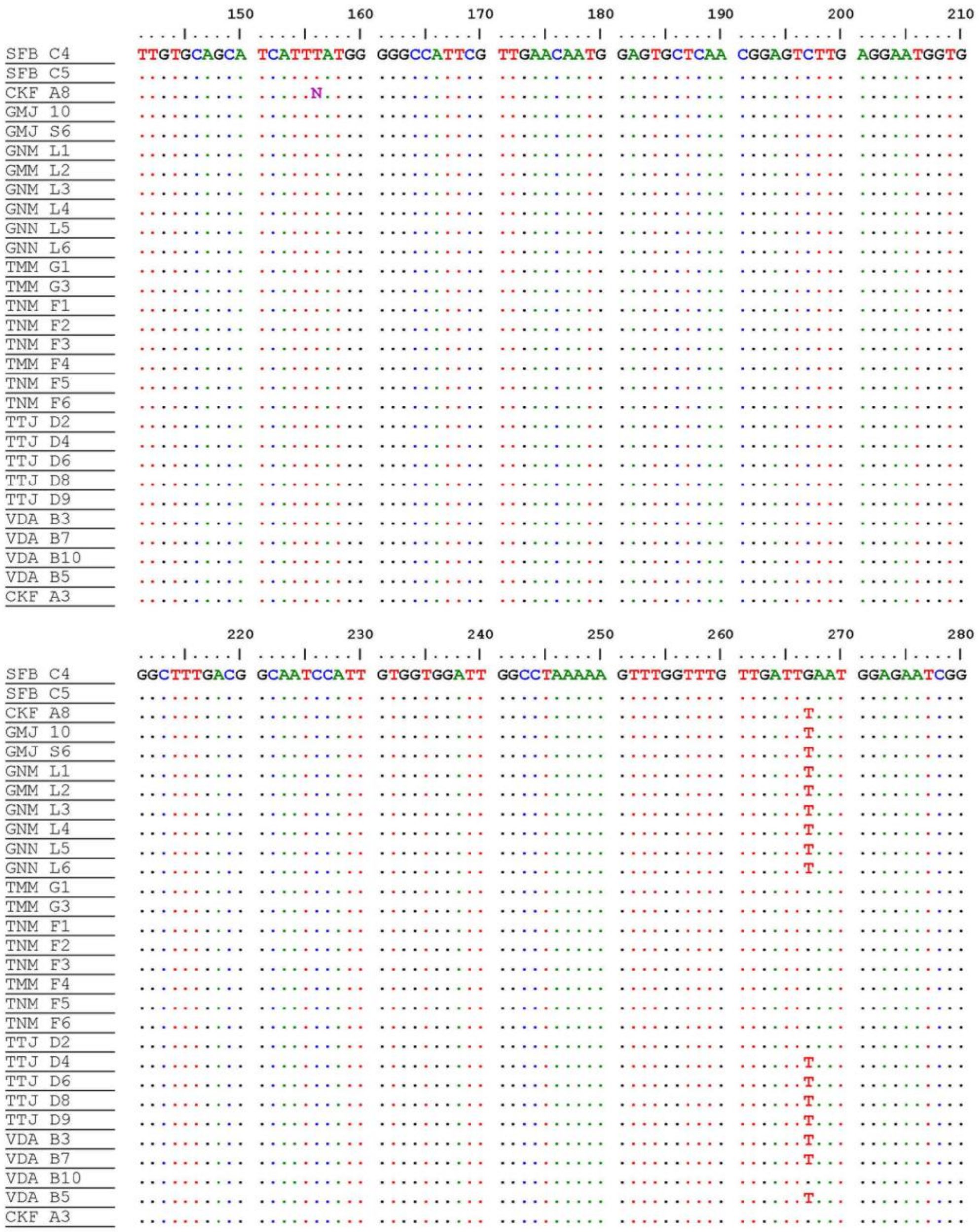
Aligned Internally Transcribed Spacer-1 sequence of all the Artemia samples



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                   150        160        170        180        190        200        210
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4     TTGTGCAGCA TCATTTATGG GGGCCATTCG TTGAACAATG GAGTGCTCAA CGGAGTCTTG AGGAATGGTG
SFB C5     .......... .......... .......... .......... .......... .......... ..........
CKF A8     .......... ......N... .......... .......... .......... .......... ..........
GMJ 10     .......... .......... .......... .......... .......... .......... ..........
GMJ S6     .......... .......... .......... .......... .......... .......... ..........
GNM L1     .......... .......... .......... .......... .......... .......... ..........
GMM L2     .......... .......... .......... .......... .......... .......... ..........
GNM L3     .......... .......... .......... .......... .......... .......... ..........
GNM L4     .......... .......... .......... .......... .......... .......... ..........
GNN L5     .......... .......... .......... .......... .......... .......... ..........
GNN L6     .......... .......... .......... .......... .......... .......... ..........
TMM G1     .......... .......... .......... .......... .......... .......... ..........
TMM G3     .......... .......... .......... .......... .......... .......... ..........
TNM F1     .......... .......... .......... .......... .......... .......... ..........
TNM F2     .......... .......... .......... .......... .......... .......... ..........
TNM F3     .......... .......... .......... .......... .......... .......... ..........
TMM F4     .......... .......... .......... .......... .......... .......... ..........
TNM F5     .......... .......... .......... .......... .......... .......... ..........
TNM F6     .......... .......... .......... .......... .......... .......... ..........
TTJ D2     .......... .......... .......... .......... .......... .......... ..........
TTJ D4     .......... .......... .......... .......... .......... .......... ..........
TTJ D6     .......... .......... .......... .......... .......... .......... ..........
TTJ D8     .......... .......... .......... .......... .......... .......... ..........
TTJ D9     .......... .......... .......... .......... .......... .......... ..........
VDA B3     .......... .......... .......... .......... .......... .......... ..........
VDA B7     .......... .......... .......... .......... .......... .......... ..........
VDA B10    .......... .......... .......... .......... .......... .......... ..........
VDA B5     .......... .......... .......... .......... .......... .......... ..........
CKF A3     .......... .......... .......... .......... .......... .......... ..........

                   220        230        240        250        260        270        280
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4     GGCTTTGACG GCAATCCATT GTGGTGGATT GGCCTAAAAA GTTTGGTTTG TTGATTGAAT GGAGAATCGG
SFB C5     .......... .......... .......... .......... .......... .......... ..........
CKF A8     .......... .......... .......... .......... .......... ......T... ..........
GMJ 10     .......... .......... .......... .......... .......... ......T... ..........
GMJ S6     .......... .......... .......... .......... .......... ......T... ..........
GNM L1     .......... .......... .......... .......... .......... ......T... ..........
GMM L2     .......... .......... .......... .......... .......... ......T... ..........
GNM L3     .......... .......... .......... .......... .......... ......T... ..........
GNM L4     .......... .......... .......... .......... .......... ......T... ..........
GNN L5     .......... .......... .......... .......... .......... ......T... ..........
GNN L6     .......... .......... .......... .......... .......... ......T... ..........
TMM G1     .......... .......... .......... .......... .......... .......... ..........
TMM G3     .......... .......... .......... .......... .......... .......... ..........
TNM F1     .......... .......... .......... .......... .......... .......... ..........
TNM F2     .......... .......... .......... .......... .......... .......... ..........
TNM F3     .......... .......... .......... .......... .......... .......... ..........
TMM F4     .......... .......... .......... .......... .......... .......... ..........
TNM F5     .......... .......... .......... .......... .......... .......... ..........
TNM F6     .......... .......... .......... .......... .......... .......... ..........
TTJ D2     .......... .......... .......... .......... .......... .......... ..........
TTJ D4     .......... .......... .......... .......... .......... ......T... ..........
TTJ D6     .......... .......... .......... .......... .......... ......T... ..........
TTJ D8     .......... .......... .......... .......... .......... ......T... ..........
TTJ D9     .......... .......... .......... .......... .......... ......T... ..........
VDA B3     .......... .......... .......... .......... .......... ......T... ..........
VDA B7     .......... .......... .......... .......... .......... ......T... ..........
VDA B10    .......... .......... .......... .......... .......... .......... ..........
VDA B5     .......... .......... .......... .......... .......... ......T... ..........
CKF A3     .......... .......... .......... .......... .......... .......... ..........
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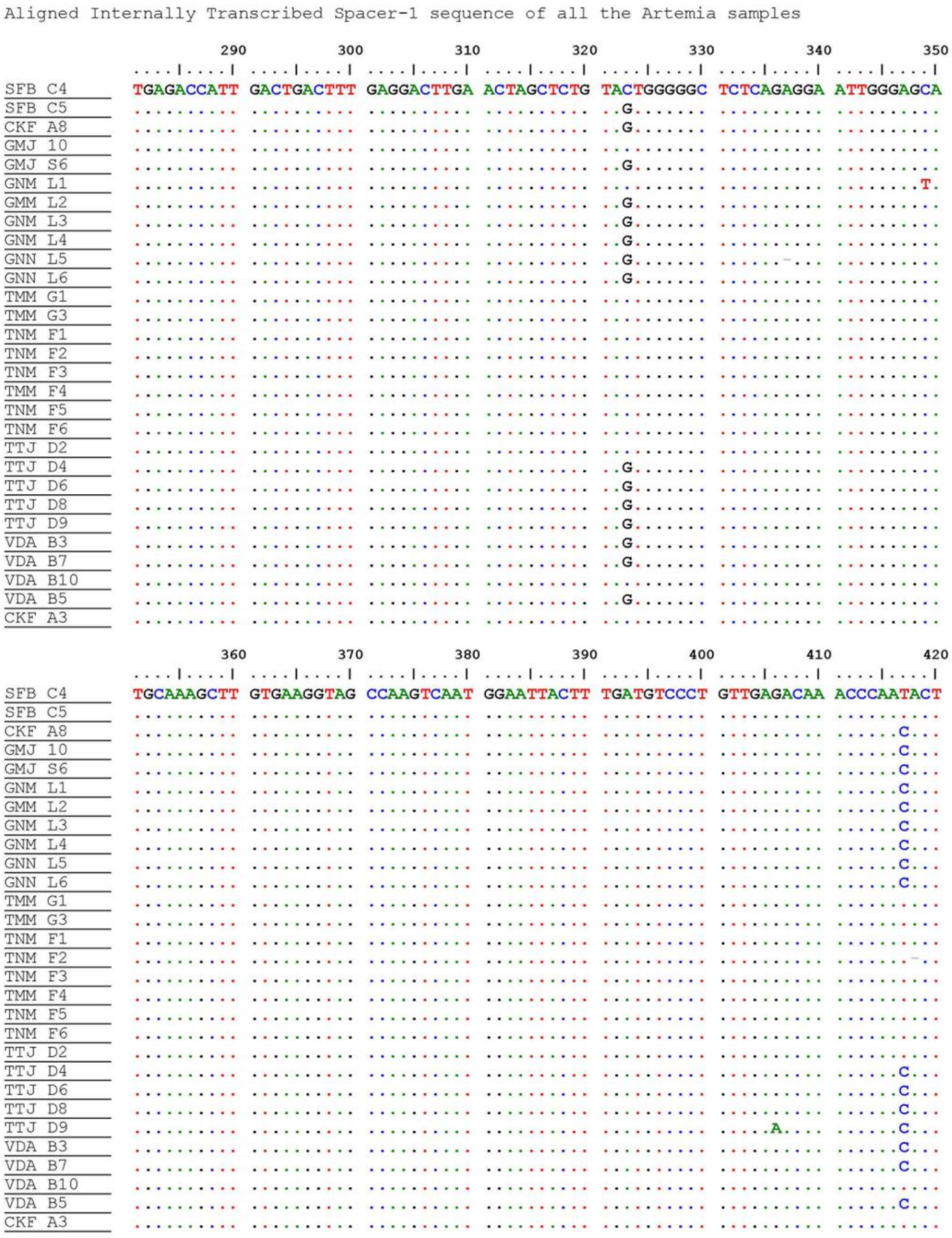

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Aligned Internally Transcribed Spacer-1 sequence of all the Artemia samples

                  290        300        310        320        330        340        350
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4     TGAGACCATT GACTGACTTT GAGGACTTGA ACTAGCTCTG TACTGGGGGC TCTCAGAGGA ATTGGGAGCA
SFB C5     .......... .......... .......... .......... ..G....... .......... ..........
CKF A8     .......... .......... .......... .......... ..G....... .......... ..........
GMJ 10     .......... .......... .......... .......... .......... .......... ..........
GMJ S6     .......... .......... .......... .......... ..G....... .......... ..........
GNM L1     .......... .......... .......... .......... .......... .......... ........T.
GMM L2     .......... .......... .......... .......... ..G....... .......... ..........
GNM L3     .......... .......... .......... .......... ..G....... .......... ..........
GNM L4     .......... .......... .......... .......... ..G....... .......... ..........
GNN L5     .......... .......... .......... .......... ..G....... ......-... ..........
GNN L6     .......... .......... .......... .......... ..G....... .......... ..........
TMM G1     .......... .......... .......... .......... .......... .......... ..........
TMM G3     .......... .......... .......... .......... .......... .......... ..........
TNM F1     .......... .......... .......... .......... .......... .......... ..........
TNM F2     .......... .......... .......... .......... .......... .......... ..........
TNM F3     .......... .......... .......... .......... .......... .......... ..........
TMM F4     .......... .......... .......... .......... .......... .......... ..........
TNM F5     .......... .......... .......... .......... .......... .......... ..........
TNM F6     .......... .......... .......... .......... .......... .......... ..........
TTJ D2     .......... .......... .......... .......... .......... .......... ..........
TTJ D4     .......... .......... .......... .......... ..G....... .......... ..........
TTJ D6     .......... .......... .......... .......... ..G....... .......... ..........
TTJ D8     .......... .......... .......... .......... ..G....... .......... ..........
TTJ D9     .......... .......... .......... .......... ..G....... .......... ..........
VDA B3     .......... .......... .......... .......... ..G....... .......... ..........
VDA B7     .......... .......... .......... .......... ..G....... .......... ..........
VDA B10    .......... .......... .......... .......... .......... .......... ..........
VDA B5     .......... .......... .......... .......... ..G....... .......... ..........
CKF A3     .......... .......... .......... .......... .......... .......... ..........

                  360        370        380        390        400        410        420
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4     TGCAAAGCTT GTGAAGGTAG CCAAGTCAAT GGAATTACTT TGATGTCCCT GTTGAGACAA ACCCAATACT
SFB C5     .......... .......... .......... .......... .......... .......... ..........
CKF A8     .......... .......... .......... .......... .......... .......... ......C...
GMJ 10     .......... .......... .......... .......... .......... .......... ......C...
GMJ S6     .......... .......... .......... .......... .......... .......... ......C...
GNM L1     .......... .......... .......... .......... .......... .......... ......C...
GMM L2     .......... .......... .......... .......... .......... .......... ......C...
GNM L3     .......... .......... .......... .......... .......... .......... ......C...
GNM L4     .......... .......... .......... .......... .......... .......... ......C...
GNN L5     .......... .......... .......... .......... .......... .......... ......C...
GNN L6     .......... .......... .......... .......... .......... .......... ......C...
TMM G1     .......... .......... .......... .......... .......... .......... ..........
TMM G3     .......... .......... .......... .......... .......... .......... ..........
TNM F1     .......... .......... .......... .......... .......... .......... ..........
TNM F2     .......... .......... .......... .......... .......... .......... .......-..
TNM F3     .......... .......... .......... .......... .......... .......... ..........
TMM F4     .......... .......... .......... .......... .......... .......... ..........
TNM F5     .......... .......... .......... .......... .......... .......... ..........
TNM F6     .......... .......... .......... .......... .......... .......... ..........
TTJ D2     .......... .......... .......... .......... .......... .......... ..........
TTJ D4     .......... .......... .......... .......... .......... .......... ......C...
TTJ D6     .......... .......... .......... .......... .......... .......... ......C...
TTJ D8     .......... .......... .......... .......... .......... .......... ......C...
TTJ D9     .......... .......... .......... .......... .......... .....A.... ......C...
VDA B3     .......... .......... .......... .......... .......... .......... ......C...
VDA B7     .......... .......... .......... .......... .......... .......... ......C...
VDA B10    .......... .......... .......... .......... .......... .......... ..........
VDA B5     .......... .......... .......... .......... .......... .......... ......C...
CKF A3     .......... .......... .......... .......... .......... .......... ..........
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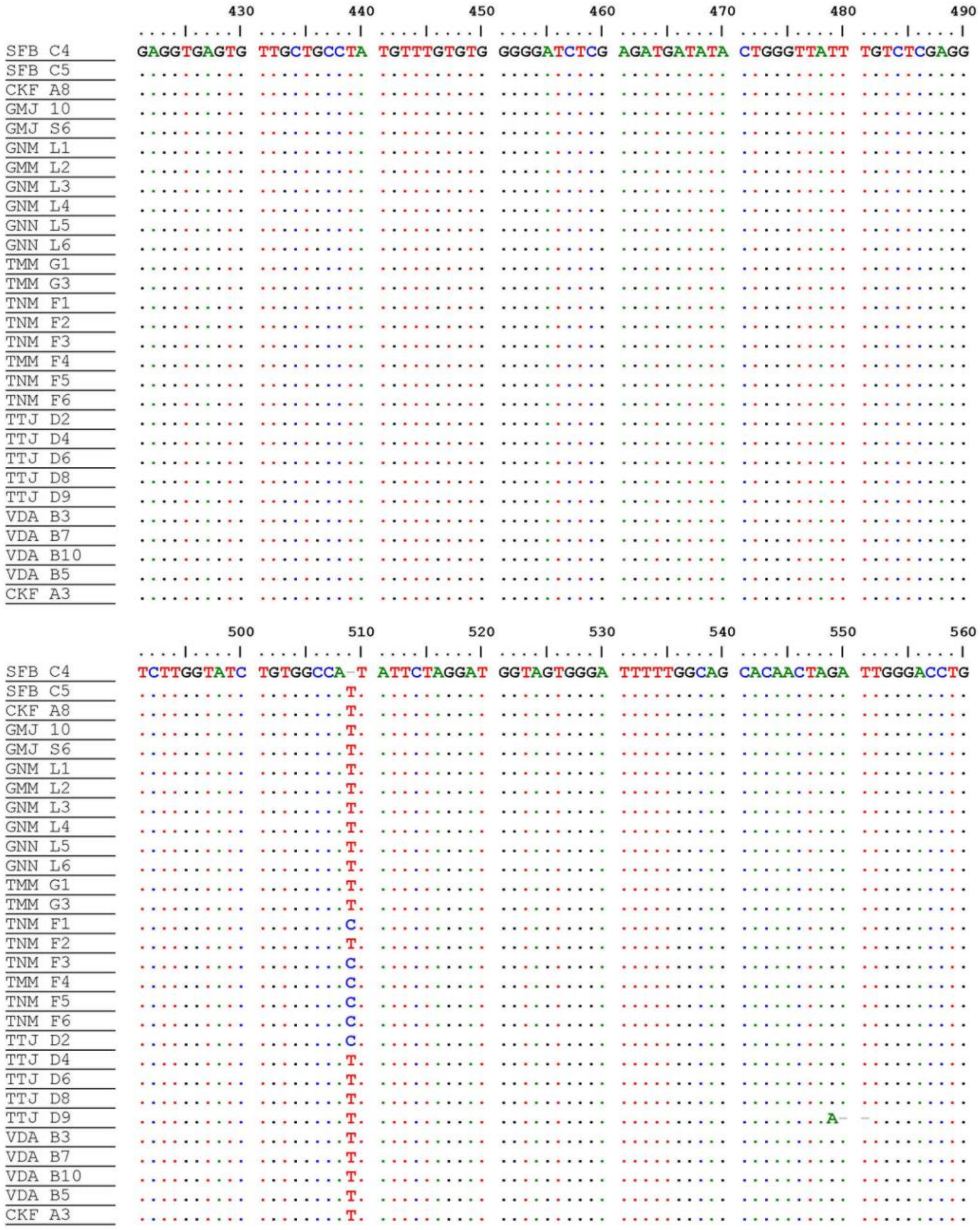
Aligned Internally Transcribed Spacer-1 sequence of all the Artemia samples



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                  430        440        450        460        470        480        490
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4    GAGGTGAGTG TTGCTGCCTA TGTTTGTGTG GGGGATCTCG AGATGATATA CTGGGTTATT TGTCTCGAGG
SFB C5    .......... .......... .......... .......... .......... .......... ..........
CKF A8    .......... .......... .......... .......... .......... .......... ..........
GMJ 10    .......... .......... .......... .......... .......... .......... ..........
GMJ S6    .......... .......... .......... .......... .......... .......... ..........
GNM L1    .......... .......... .......... .......... .......... .......... ..........
GMM L2    .......... .......... .......... .......... .......... .......... ..........
GNM L3    .......... .......... .......... .......... .......... .......... ..........
GNM L4    .......... .......... .......... .......... .......... .......... ..........
GNN L5    .......... .......... .......... .......... .......... .......... ..........
GNN L6    .......... .......... .......... .......... .......... .......... ..........
TMM G1    .......... .......... .......... .......... .......... .......... ..........
TMM G3    .......... .......... .......... .......... .......... .......... ..........
TNM F1    .......... .......... .......... .......... .......... .......... ..........
TNM F2    .......... .......... .......... .......... .......... .......... ..........
TNM F3    .......... .......... .......... .......... .......... .......... ..........
TMM F4    .......... .......... .......... .......... .......... .......... ..........
TNM F5    .......... .......... .......... .......... .......... .......... ..........
TNM F6    .......... .......... .......... .......... .......... .......... ..........
TTJ D2    .......... .......... .......... .......... .......... .......... ..........
TTJ D4    .......... .......... .......... .......... .......... .......... ..........
TTJ D6    .......... .......... .......... .......... .......... .......... ..........
TTJ D8    .......... .......... .......... .......... .......... .......... ..........
TTJ D9    .......... .......... .......... .......... .......... .......... ..........
VDA B3    .......... .......... .......... .......... .......... .......... ..........
VDA B7    .......... .......... .......... .......... .......... .......... ..........
VDA B10   .......... .......... .......... .......... .......... .......... ..........
VDA B5    .......... .......... .......... .......... .......... .......... ..........
CKF A3    .......... .......... .......... .......... .......... .......... ..........

                  500        510        520        530        540        550        560
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4    TCTTGGTATC TGTGGCCA-T ATTCTAGGAT GGTAGTGGGA TTTTTGGCAG CACAACTAGA TTGGGACCTG
SFB C5    .......... ........T. .......... .......... .......... .......... ..........
CKF A8    .......... ........T. .......... .......... .......... .......... ..........
GMJ 10    .......... ........T. .......... .......... .......... .......... ..........
GMJ S6    .......... ........T. .......... .......... .......... .......... ..........
GNM L1    .......... ........T. .......... .......... .......... .......... ..........
GMM L2    .......... ........T. .......... .......... .......... .......... ..........
GNM L3    .......... ........T. .......... .......... .......... .......... ..........
GNM L4    .......... ........T. .......... .......... .......... .......... ..........
GNN L5    .......... ........T. .......... .......... .......... .......... ..........
GNN L6    .......... ........T. .......... .......... .......... .......... ..........
TMM G1    .......... ........T. .......... .......... .......... .......... ..........
TMM G3    .......... ........T. .......... .......... .......... .......... ..........
TNM F1    .......... ........C. .......... .......... .......... .......... ..........
TNM F2    .......... ........T. .......... .......... .......... .......... ..........
TNM F3    .......... ........C. .......... .......... .......... .......... ..........
TMM F4    .......... ........C. .......... .......... .......... .......... ..........
TNM F5    .......... ........C. .......... .......... .......... .......... ..........
TNM F6    .......... ........C. .......... .......... .......... .......... ..........
TTJ D2    .......... ........C. .......... .......... .......... .......... ..........
TTJ D4    .......... ........T. .......... .......... .......... .......... ..........
TTJ D6    .......... ........T. .......... .......... .......... .......... ..........
TTJ D8    .......... ........T. .......... .......... .......... .......... ..........
TTJ D9    .......... ........T. .......... .......... .......... ........A- -.........
VDA B3    .......... ........T. .......... .......... .......... .......... ..........
VDA B7    .......... ........T. .......... .......... .......... .......... ..........
VDA B10   .......... ........T. .......... .......... .......... .......... ..........
VDA B5    .......... ........T. .......... .......... .......... .......... ..........
CKF A3    .......... ........T. .......... .......... .......... .......... ..........
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Aligned Internally Transcribed Spacer-1 sequence of all the Artemia samples

```
                 570        580        590        600        610        620        630
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4    CGCTTTTGCA GACGGGGGCT CTTGTGAGGA AACGAGAGCC TGCAAAGCTT GTGACACATC TTGAGTTGTG
SFB C5    .......... .......... .......... .......... .......... .......... ..........
CKF A8    .......... .......... .......... .......... .......... .......... ..........
GMJ 10    .-........ .......... .......... .......... .......... .......... ..........
GMJ S6    .......... .......... .......... .......... .......... .......... ..........
GNM L1    .......... .......... .......... .......... .......... .......... ..........
GMM L2    .......... .......... .......... .......... .......... .......... ..........
GNM L3    .......... .......... .......... .......... .......... .......... ..........
GNM L4    .......... .......... .......... .......... .......... .......... ..........
GNN L5    .......... .......... .......... .......... .......... .......... ..........
GNN L6    .......... .......... .......... .......... .......... .......... ..........
TMM G1    .......... .......... .......... .......... .......... .......... ..........
TMM G3    .......... .......... .......... .......... .......... .......... ..........
TNM F1    .......... .......... .......... .......... .......... .......... ..........
TNM F2    .......... .......... .......... .......... .......... .......... ..........
TNM F3    .......... .......... .......... .......... .......... .......... ..........
TMM F4    .......... .......... .......... .......... .......... .......... ..........
TNM F5    .......... .......... .......... .......... .......... .......... ..........
TNM F6    .......... .......... .......... .......... .......... .......... ..........
TTJ D2    .......... .......... .......... .......... .......... .......... ..........
TTJ D4    .......... .......... .......... .......... .......... .......... ..........
TTJ D6    .......... .......... .......... .......... .......... .......... ..........
TTJ D8    .......... .......... .......... .......... .......... .......... ..........
TTJ D9    .......... .......... .......... .......... .......... .......... ..........
VDA B3    .......... .......... .......... .......... .......... .......... ..........
VDA B7    .......... .......... .......... .......... .......... .......... ..........
VDA B10   .......... .......... .......... .......... .......... .......... ..........
VDA B5    .......... .......... .......... .......... .......... .......... ..........
CKF A3    .......... .......... .......... .......... .......... .......... ..........

                 640        650        660        670        680        690        700
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4    TACGAGGGCC ACAAAGTCAT GCCATCTGAA AAATAATGGA CATTAGAGTA CTAACACTGT GGTAGTTGCT
SFB C5    .......... .......... .......... .......... .......... .......... ..........
CKF A8    .......... .......... .......... .......... .......T.. .......... ..........
GMJ 10    .......... .......... .......... .......... .......T.. .......... ..........
GMJ S6    .......... .......... .......... .......... .......T.. .......... ..........
GNM L1    .......... .......... .......... .......... .......T.. .......... ..........
GMM L2    .......... .......... .......... .......... .......T.. .......... .........-
GNM L3    .......... .......... .......... .......... .......T.. .......... ..........
GNM L4    .......... .......... .......... .......... .......T.. .......... ..........
GNN L5    .......... .......... .......... .......... .......T.. .......... ........T.
GNN L6    .......... .......... .......... .......... .......T.. .......... ..........
TMM G1    .......... .......... .......... .......... .......... .......... ..........
TMM G3    .......... .......... .......... .......... .......... .......... ..........
TNM F1    .......... .......... .......... .......... .......... .......... ..........
TNM F2    .......... .......... .......... .......... .......... .......... ..........
TNM F3    .......... .......... .......... .......... .......... .......... ..........
TMM F4    .......... .......... .......... .......... .......... .......... ..........
TNM F5    .......... .......... .......... .......... .......... .......... ..........
TNM F6    .......... .......... .......... .......... .......... .......... ..........
TTJ D2    .......... .......... .......... .......... .......... .......... ..........
TTJ D4    .......... .......... .......... .......... .......T.. .......... ..........
TTJ D6    .......... .......... .......... .......... .......T.. .......... ..........
TTJ D8    .......... .......... .......... .......... .......T.. .......... ..........
TTJ D9    .......... .......... .......... .......... .......T.. .......... ..........
VDA B3    .......... .......... .......... .......... .......T.. .......... ..........
VDA B7    .......... .......... .......... .......... .......T.. .......... ..........
VDA B10   .......... .......... .......... .......... .......T.. .......... ..........
VDA B5    .......... .......... .....-.... .......... .......T.. .......... ..........
CKF A3    .......... .......... .......... .......... .......T.. .......... ..........
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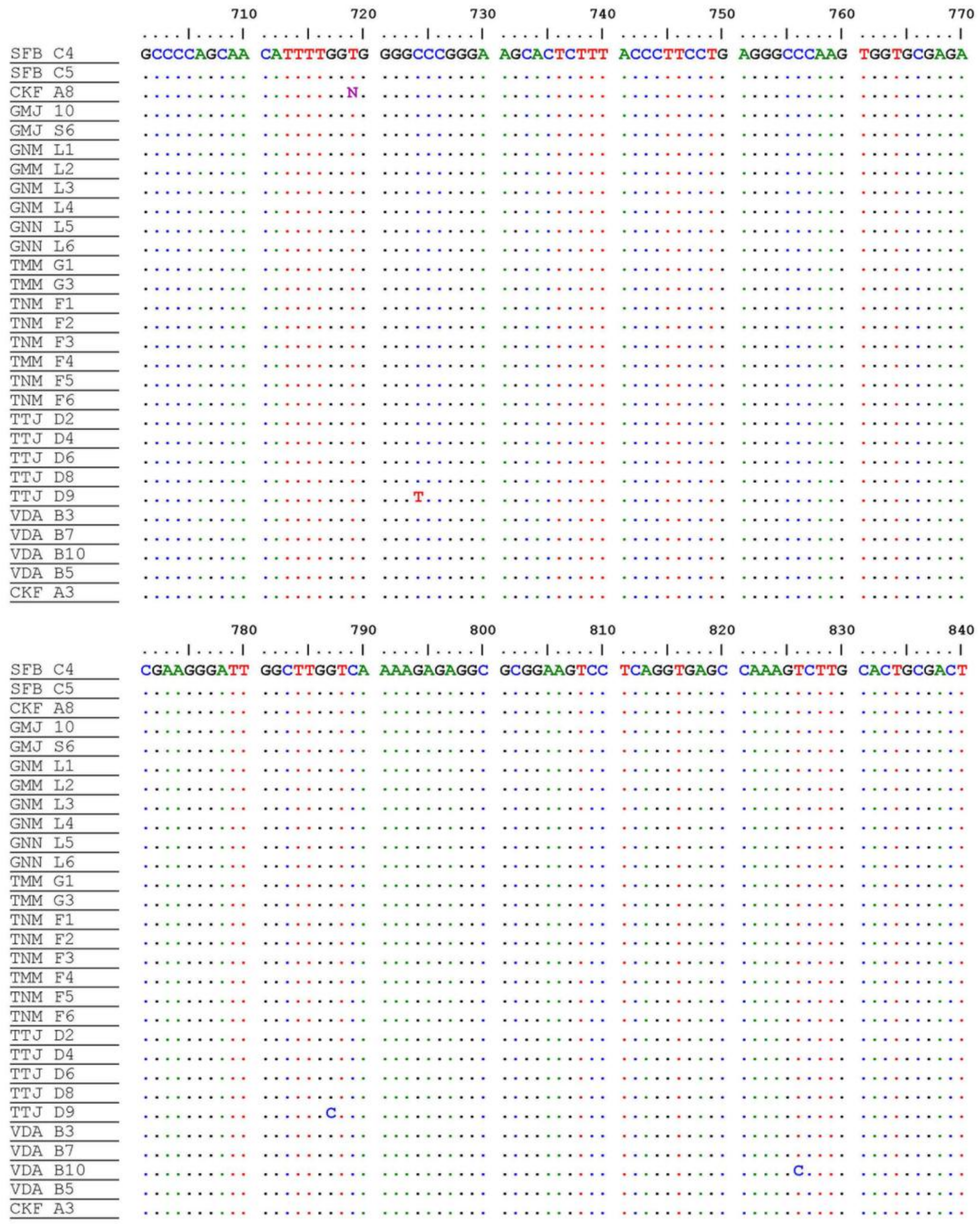
Aligned Internally Transcribed Spacer-1 sequence of all the Artemia samples



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                   710        720        730        740        750        760        770
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4    GCCCCAGCAA CATTTTGGTG GGGCCCGGGA AGCACTCTTT ACCCTTCCTG AGGGCCCAAG TGGTGCGAGA
SFB C5    .......... .......... .......... .......... .......... .......... ..........
CKF A8    .......... ........N. .......... .......... .......... .......... ..........
GMJ 10    .......... .......... .......... .......... .......... .......... ..........
GMJ S6    .......... .......... .......... .......... .......... .......... ..........
GNM L1    .......... .......... .......... .......... .......... .......... ..........
GMM L2    .......... .......... .......... .......... .......... .......... ..........
GNM L3    .......... .......... .......... .......... .......... .......... ..........
GNM L4    .......... .......... .......... .......... .......... .......... ..........
GNN L5    .......... .......... .......... .......... .......... .......... ..........
GNN L6    .......... .......... .......... .......... .......... .......... ..........
TMM G1    .......... .......... .......... .......... .......... .......... ..........
TMM G3    .......... .......... .......... .......... .......... .......... ..........
TNM F1    .......... .......... .......... .......... .......... .......... ..........
TNM F2    .......... .......... .......... .......... .......... .......... ..........
TNM F3    .......... .......... .......... .......... .......... .......... ..........
TMM F4    .......... .......... .......... .......... .......... .......... ..........
TNM F5    .......... .......... .......... .......... .......... .......... ..........
TNM F6    .......... .......... .......... .......... .......... .......... ..........
TTJ D2    .......... .......... .......... .......... .......... .......... ..........
TTJ D4    .......... .......... .......... .......... .......... .......... ..........
TTJ D6    .......... .......... .......... .......... .......... .......... ..........
TTJ D8    .......... .......... .......... .......... .......... .......... ..........
TTJ D9    .......... .......... ...T...... .......... .......... .......... ..........
VDA B3    .......... .......... .......... .......... .......... .......... ..........
VDA B7    .......... .......... .......... .......... .......... .......... ..........
VDA B10   .......... .......... .......... .......... .......... .......... ..........
VDA B5    .......... .......... .......... .......... .......... .......... ..........
CKF A3    .......... .......... .......... .......... .......... .......... ..........

                   780        790        800        810        820        830        840
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4    CGAAGGGATT GGCTTGGTCA AAAGAGAGGC GCGGAAGTCC TCAGGTGAGC CAAAGTCTTG CACTGCGACT
SFB C5    .......... .......... .......... .......... .......... .......... ..........
CKF A8    .......... .......... .......... .......... .......... .......... ..........
GMJ 10    .......... .......... .......... .......... .......... .......... ..........
GMJ S6    .......... .......... .......... .......... .......... .......... ..........
GNM L1    .......... .......... .......... .......... .......... .......... ..........
GMM L2    .......... .......... .......... .......... .......... .......... ..........
GNM L3    .......... .......... .......... .......... .......... .......... ..........
GNM L4    .......... .......... .......... .......... .......... .......... ..........
GNN L5    .......... .......... .......... .......... .......... .......... ..........
GNN L6    .......... .......... .......... .......... .......... .......... ..........
TMM G1    .......... .......... .......... .......... .......... .......... ..........
TMM G3    .......... .......... .......... .......... .......... .......... ..........
TNM F1    .......... .......... .......... .......... .......... .......... ..........
TNM F2    .......... .......... .......... .......... .......... .......... ..........
TNM F3    .......... .......... .......... .......... .......... .......... ..........
TMM F4    .......... .......... .......... .......... .......... .......... ..........
TNM F5    .......... .......... .......... .......... .......... .......... ..........
TNM F6    .......... .......... .......... .......... .......... .......... ..........
TTJ D2    .......... .......... .......... .......... .......... .......... ..........
TTJ D4    .......... .......... .......... .......... .......... .......... ..........
TTJ D6    .......... .......... .......... .......... .......... .......... ..........
TTJ D8    .......... .......... .......... .......... .......... .......... ..........
TTJ D9    .......... ......C... .......... .......... .......... .......... ..........
VDA B3    .......... .......... .......... .......... .......... .......... ..........
VDA B7    .......... .......... .......... .......... .......... .......... ..........
VDA B10   .......... .......... .......... .......... .......... .....C.... ..........
VDA B5    .......... .......... .......... .......... .......... .......... ..........
CKF A3    .......... .......... .......... .......... .......... .......... ..........
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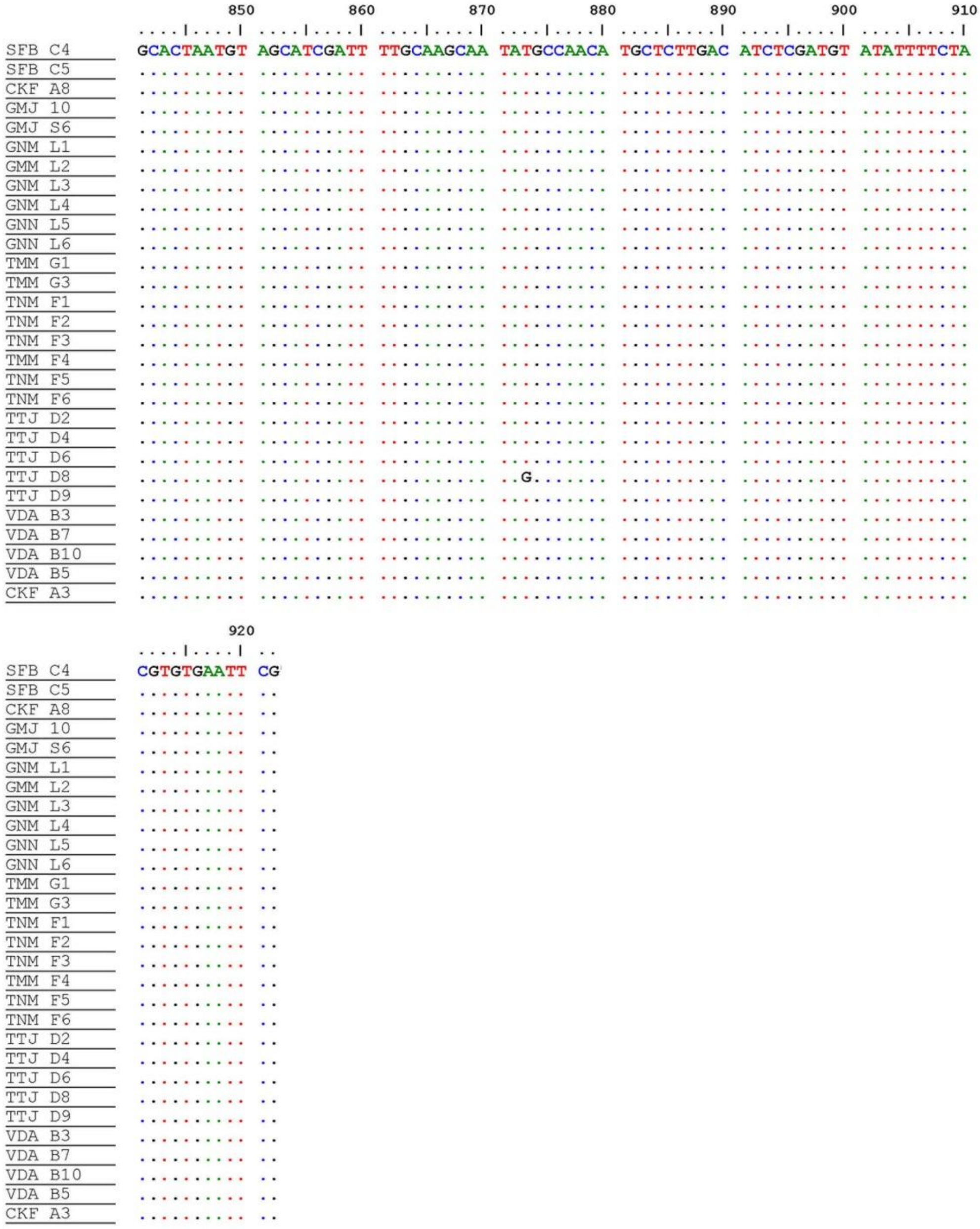
Aligned Internally Transcribed Spacer-1 sequence of all the Artemia samples



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                    850        860        870        880        890        900        910
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
SFB C4     GCACTAATGT AGCATCGATT TTGCAAGCAA TATGCCAACA TGCTCTTGAC ATCTCGATGT ATATTTTCTA
SFB C5     .......... .......... .......... .......... .......... .......... ..........
CKF A8     .......... .......... .......... .......... .......... .......... ..........
GMJ 10     .......... .......... .......... .......... .......... .......... ..........
GMJ S6     .......... .......... .......... .......... .......... .......... ..........
GNM L1     .......... .......... .......... .......... .......... .......... ..........
GMM L2     .......... .......... .......... .......... .......... .......... ..........
GNM L3     .......... .......... .......... .......... .......... .......... ..........
GNM L4     .......... .......... .......... .......... .......... .......... ..........
GNN L5     .......... .......... .......... .......... .......... .......... ..........
GNN L6     .......... .......... .......... .......... .......... .......... ..........
TMM G1     .......... .......... .......... .......... .......... .......... ..........
TMM G3     .......... .......... .......... .......... .......... .......... ..........
TNM F1     .......... .......... .......... .......... .......... .......... ..........
TNM F2     .......... .......... .......... .......... .......... .......... ..........
TNM F3     .......... .......... .......... .......... .......... .......... ..........
TMM F4     .......... .......... .......... .......... .......... .......... ..........
TNM F5     .......... .......... .......... .......... .......... .......... ..........
TNM F6     .......... .......... .......... .......... .......... .......... ..........
TTJ D2     .......... .......... .......... .......... .......... .......... ..........
TTJ D4     .......... .......... .......... .......... .......... .......... ..........
TTJ D6     .......... .......... .......... .......... .......... .......... ..........
TTJ D8     .......... .......... .......... ..G....... .......... .......... ..........
TTJ D9     .......... .......... .......... .......... .......... .......... ..........
VDA B3     .......... .......... .......... .......... .......... .......... ..........
VDA B7     .......... .......... .......... .......... .......... .......... ..........
VDA B10    .......... .......... .......... .......... .......... .......... ..........
VDA B5     .......... .......... .......... .......... .......... .......... ..........
CKF A3     .......... .......... .......... .......... .......... .......... ..........

                    920
           ....|....| ..
SFB C4     CGTGTGAATT CG
SFB C5     .......... ..
CKF A8     .......... ..
GMJ 10     .......... ..
GMJ S6     .......... ..
GNM L1     .......... ..
GMM L2     .......... ..
GNM L3     .......... ..
GNM L4     .......... ..
GNN L5     .......... ..
GNN L6     .......... ..
TMM G1     .......... ..
TMM G3     .......... ..
TNM F1     .......... ..
TNM F2     .......... ..
TNM F3     .......... ..
TMM F4     .......... ..
TNM F5     .......... ..
TNM F6     .......... ..
TTJ D2     .......... ..
TTJ D4     .......... ..
TTJ D6     .......... ..
TTJ D8     .......... ..
TTJ D9     .......... ..
VDA B3     .......... ..
VDA B7     .......... ..
VDA B10    .......... ..
VDA B5     .......... ..
CKF A3     .......... ..
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