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Embryogenesis of *Marsupenaeus japonicus* (Bate, 1888) under normal and treated conditions with emphasis on the localization of the neuropeptide, RF-amide during larval metamorphosis in normal condition

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Abstract

Shrimps of *Marsupenaeus japonicus* were collected from the inshore waters of the Arabian Gulf of the Ad-dammam estuary from June to September 2017. Male shrimps were subjected to electrical stimuli in 9 volt (V) direct current applied by means of electrodes. Spermatophores extruded were injected anterior to the thelycum of females. Each female was placed in well aerated sea water tanks for ≈ 24 hours. Larval stages were collected from the tanks and were the main studying material. The embryonic stages were described and larvae were studied under normal and treated conditions. MF 2.5 mM, C8:0 1 µM, Asymmetric dimethylarginine (ADMA) 2.5 mM, and Acetyl choline 0.5 mM accelerated larval transformation from nauplius to early mysis stages. Serotonin 10 µg/ml and Acetyl choline 1 mM exerted a negative effect while C8:0 0.1 or 10 µM, MF 300 µM, Asymmetric dimethylarginine (ADMA) 1 mM and 1.5 mM did not affect larval transformation. Laval transformation was negatively affected by the lipid-regulating agents Atrovastatin & Pravastatin, the antihistaminic agent Cetrizine hydrochloride, the β-blocker Nebivolol & Atenelol and the calcium-channel blocker Amlodipine Besylate. Expression of RF-amide neuropeptide was followed during larval stages. The effect of these bioactive inducers cannot be followed after early mysis stage because of the high percentage of mortality. Napluii

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and zoeae showed that RF-amide neuropeptide was mantled in the brain and peripheral neurons. This neuropeptide diffused from the brain and spread in the peripheral neurons of postlarval stages.

Keywords *Marsupenaeus japonicus* – embryonic stages - larvae – pharmacological inducers – lipid -regulating agents- RF-amide neuropeptide

INTRODUCTION

Aquaculture is practiced worldwide to cover mainly fish (pisciculture); shrimp (shrimp farming); frogs and molluscs: oysters and mussels (malacoculture). Increasing productivity of the shrimp *Marsupenaeus japonicus* in aquaculture constitutes a subsidy for the induction of growth rates and fecundity. Accompaniments production process of decapod larvae in captivity have been shown in many studies (Daniels, et al. 1995; Ra'anan et al. 1991; Ra'Anan & Cohen, 1985; Sandifer et al. 1984). Shrimp farming has spread rapidly in many countries such as KSA, UAE; USA, China and Australia by adapting the developed clear water technology and regulated conditions. Investigations on larval development and morphological description of the different stages and sub-stages of the main decapod crustacean species have been addressed by the authors (Harris & Sandifer, 1986; Lemaire & Marcellini,2003;Sandifer & Smith, 2009). The first stages were fulfilled with the purpose of knowing the life cycle of the species under study, recognizing their presence in the plankton samples and being able to estimate the abundance (Ra'Anan & Cohen,1985; Hertzler & Clark, 1992). The characteristics of the larvae, in turn, made it possible to support phylogenetic interpretations and links between the different strains of Decapoda under study (Daniels, et al. 1995; Subasinghe, 2001). Shrimps can be cultivated in the laboratory from gravid females that can be obtained from commercial fishing area. In the coastal waters of Saudi Arabia gravid females are obtainable during the sexual maturity period, between Jule and October (Gaber, et al. 2018). The method of cultivation is the same as that used in the laboratory stage, in UAE, in USA, in Japan, and other countries that are engaged in the breeding of commercial penaeids. Artificial insemination and insertion of spermatophores in females´ thelyca increases productivity in shrimp aquaculture (Sandifer & Smith, 2009). The kingdom of Saudi Arabia has many shrimp aquaculture farms like Red Sea shrimp farm, Saudi Fisheries Company, National Aquaculture Group (NAQUA) which is one of the largest aquaculture operations in Saudi Arabia with 16 shrimp farms. The electro-ejaculation technique is more widespread use in male shrimps to obtain spermatophores, for being faster and providing the spermatophores for artificial fertilization. This technique was used for the first time in *M. rosenbergii* by Sandifer, et al. (1984) and (Harris and Sandifer, 1986; Sandifer & Smith, 2009). The larval development among penaeids is species dependent (Subasinghe, 2001). It relies also on habitat and abiotic conditions (Sandifer, et al. 1984). The days of larval development can be shortened with slightly higher temperatures. On the other hand, in aquaculture the mortality in the initial zoea stage can be controlled and diminished with adequate food and better environmental conditions (Wickins & Beard, 1974;

Daniels, et al. 1995). In shrimp farming the tanks of larviculture are very varied, being circular of flat bottom, circular conical bottoms wooden tanks coated with plastic, etc .; but the most practical are those rectangular because small or large-scale larvae can be adapted (Subasinghe, 2001).

The nervous system forms the basis for information transfer and responsible for metamorphosis within shrimps (Lemaire & Marcellini, 2003). The planula larva of the hydroid *Clava multicornis* (Forskål, 1775) has a complex nervous system, characterized by the presence of distinct, anteriorly concentrated peptidergic populations of amidated neurons, presumably involved in the detection of environmental stimuli and metamorphic signals. Differently from other hydrozoan larvae in *C. multicornis* planulae GLW-positive cells with putative sensory role have a peculiar dome-shaped forefront organization, followed by a belt of RF-positive nerve cells. By immunohistochemistry, (Pennati, et al. 2013) investigated the transformation of the peptidergic (GLW-amide and RF-amide) larval neuroanatomy at different stages of metamorphosis and the subsequent development of the primary polyp nervous system. Authors focused to study the role of neuropeptides on the larval metamorphosis (Brumwell & Martin, 2002; Grimmelikhuijzen, et al. 2002; Iwao, et al. 2002; Dockray, 2004; Pernet, et al. 2004; Hamaguchi-Hamada, et al. 2009; Grasso, et al. 2011; Pennati, et al. 2013). Other authors interested to study the messenger nitrogen monoxide (NO) and cyclic nucleotides on neuronal outgrowth and larval metamorphosis (Wu, et al. 1994;Cramer, et al. 1998; Gibbs & Truman, 1998; Froggett & Leise, 1999; Bishop & Brandhorst, 2001; Bishop, et al. 2001; Haase, 2003; Jungmann et al. 2004). Asymmetric dimethylarginine (ADMA) is an endogenously formed, competitive inhibitor of NO synthase (Shibata et al. 2008). Methylarginines are formed by the post-translational methylation of arginine residues in proteins.

The implementation of shrimp aquaculture requires planning in time and space, with the integration of a group of researchers and technicians at an interdisciplinary level to ensure the success of the company. The basic aspects that must be faced simultaneously in shrimp aquaculture are: gonadal development and fecundity of the species to be cultivated, obtain total spawning of captive females, research on phytoplankton in aquacultures in order to achieve the most suitable composition for larval feeding, study of the most convenient nutrition for the larval, post-larval, juvenile and adult stages, study of environmental conditions, related to tolerance to changes in temperature, salinity, oxygen and other variables, study of the variation of the bioactive chemical composition of larval stages that enhance their metamorphosis and the different types of water toxicants in aquaculture. The main purpose of this study was how to improve gamete production, increase fertilization density, providing a suitable medium for the developing larval stages and improve eugenic manner of the new generations. Our study tried to identify some bioactive inducers that enhance and increase larval metamorphosis and some water toxicants that hinder the cultivation in aquaculture. In this study we investigated the effects of signaling factors and neuro-transmitters on the *in vitro* transformation of larvae of *Marsupenaeus japonicus* (Bate, 1888).

MATERIAL AND METHODS

Shrimp collection

For the capture of shrimps *Marsupenaeus japonicus* , a commercial fishing boat has been used, dragging between 10 and 20 minutes with beam network (beam trawl), in order to obtain shrimps in good vital conditions. This sampling was done from the inshore waters of the Arabian Gulf of the Ad-dammam estuary from June to September 2017 (spawning period of most shrimp species in the Arabian Gulf (Gaber, et al. 2018). The females that showed the dark ovary, easily visible through the carapace, were selected and set aside in glass tanks, renewing the water to prevent the temperature from rising and continuous aeriation was supplied. Males were set aside in other glass tanks with same conditions. Powdered fish were added to the aquaria as nutrient supplement. 60 mature females and other 60 males close to spawning for each month have been selected, with weight and length ranged from 55.8 ± 11.5 g and 21.4 ± 5.5 g and 12.7 ± 1.5 cm respectively.

Heterologous insemination

Each male shrimp was submitted to a single test with electrical stimuli in 9 volt (V) based on the work of Aiken et al. (1984), on the lobsters *Homarus americanus* and Aiken, et al. (1984) and Harris & Sandifer (1986) on *M. rosenbergii.* Spermatophores extruded were used immediately as they were injected anterior to the thelycum of females between the base of the third and fourth walking legs. Pressing on spermatophores was carried out till insemination completed. The thelycum served as the seminal receptacle and was enclosed by the coxopodites of the third and fourth walking legs. Each female was placed afterwards in well aerated sea water tanks and fish powder was sullied as nutrient. These females spawned 24 hours after artificial fertilization. After ≈ 8-10 min. post laying nauplii developed. Using glass beakers of 50 ml and a micropipette, ≈ 20 hatched nauplii were placed in each beaker with sea water. The first beaker was left with sea water as control and the sea water in the other beakers was sucked and replaced with a particular pharmacological inducer with a definite concentration to test for its effect on larval transformation from nauplii to mysis stage (according to Gaber, 2002 unpublished Ph. D. thesis). Each beaker was tested at intervals for ≈ 120 hours. The pharmacological inducers were: sea water (control) ; MF (Methyl Farnesoate) (2.5 mM - 300 µM), claimed to induce larval molting and growth ; 1,2-dioctanyl-rac-glycerol (C8) (10 µM - 1 µM - 0.1 µ M) claimed to be involved in signaling systems; Serotonin (10 µg/ml) ; Acetyl choline (1 mM - 0.5 mM) ; Asymmetric dimethylarginine (ADMA) (1 mM - 1.5 mM -2.5 mM) ; claimed to serve in neuronal control (neurotransmitter). The main purpose of this technique was to provide a suitable medium necessary for the transformed larval stages and to avoid larval mortality. The inducers that enhance and accelerate larval metamorphosis can be applied later in shrimp aquaculture.

Due to marine pollution and anthropogenic activities, some chemical compounds are born in marine ecosystems and affect the general life and embryology of marine organisms. This study investigated the impact of lipid-regulating agents Atrovastatin

(5, 20 & 40 mg) & Pravastatin (50 mg & 100 mg), the antihistaminic Cetrizine hydrochloride (10 mg), the β-blocker Nebivolol (2.5mg, 5 mg. & 10 mg) & Atenelol (25 mg & 100 mg) and the calcium-channel blocker Amlodipine Besylate (2.5mg , 5.0mg , 10mg) on larval transformation from nauplii to mysis (according to Ali, et al. 2016) . All chemical agents were dissolved directly in the sea water of the cultures. Larval stages from early Zoea - early post-larva were measured with slide micrometer and tabulated (Table 3).

Immunohistochemical staining

Paraformaldehyde (4 %) was used as a fixative agent for larva stages. The samples were washed twice in 0.1 M phosphate buffer saline, four times in 0.4 M glycerol, and twice in PPTA for 15 min. After washing the samples were incubated for 12 hours in anti-RF-amide primary antibody diluted (1:200) in PPTA. Anti-RF-amid is an antibody against neuropeptides which have a C terminal amino group Arg-phe-NH2. Subsequently, the samples were washed twice in 0.1 M phosphate buffer, four times in 0.4 M glycerol, and two times in PPTA for 15 min. Incubation in secondary antibody with FITC-antibody diluted with PPTA and horse serum (1:100) was carried out for 12 hours. The samples were then washed two times in PPTA, two times in PBS (5 min), stained in 0.1 % Evans Blue for 2 min and finally washed in PBS. Larvae were embedded in 10% Glycerol in PBS and 25 mg/ml 1, 4 – DABCO. Larvae were photographed under a fluorescence ZEISS-AxioPhot microscope. The protocol used was according to (modified method of Bishop et al. 2008; Roberta, et al. 2013).

Statistical analysis of larval stages

Larvae of *Marsupenaeus japonicas* were chosen from different developmental stages and counted. Each trial was in triplicate and the mean of the of developmental stages was calculated and subjected to One-way analysis of variance.

RESULTS

Broodstock of *Marsupenaeus japonicas* under normal and treated conditions

Spermatophores were used immediately as they were injected anterior to the thelycum of females between the base of the third and fourth walking legs. Among the 60 electroejaculation tests performed, 38 were achieved, corresponding to 63.3%. Each female was placed afterwards in well aerated sea water tanks for ≈ 24 hours. Females spawned 24 hours after artificial fertilization. Fertilized and cleaved eggs were collected from all tanks. Naturally, eggs were laid in the aquaria in the form of masses with average number 35-60 eggs per mass. The ripe egg was rounded and enclosed in a flatted squamous sheath left from the oolemma and formed a fluid-filled perivitelline space (Table 1).

Table 1. Embryogenesis and larval stages of *Marsupenaeus japonicus.* Parentheses in each period mean the start-time and end-time of each stage at 20°C.

Stage	**Time after fertilization**	**% of hatching**
one cell	23 min	8%
cleavage period (2.20-3.50 hrs)		
44-cel	2 hr 20min	20%
64-cell	3 hr	25%
76-cell	3 hr 50 min	27%
gastrula Period (4.55-6.10 hrs)		
110-cell, initial gastrula	4hr 55 min	29%
early gastrula	5 hr 15 min	30%
mid gastrula	5 hr 45 min	35%
late gastrula	6 hr 10 min	37%
neurula Period (6.3-8.5 hrs)		
early neurula	6 hr 25 min	39%
mid neurula	6 hr 50 min	41%
late neurula	7 hr 30 min	45%
Nauplius larva (8.10-16.25 hrs)		
initial Nauplius	12 hr 10 min	47%
early Nauplius	16 hr 40 min	49%
late Nauplius	24 hr 10 min	52%
early Zoea	50 hr 50 min	56%
mid Zoea	80 hr 15 min	59%
late Zoea	96 hr 10 min	64%
early Mysis	120 hr 00min	70%
mid Mysis	140 hr 15 min	79%
late Mysis	200 hr 25 min	95%
larva Period (18.20 – 50.55 hrs)		
early post-larva	350 hr 20 min	98%

The ooplasm contained a homogenous centrally distributed yolk granule. It had a diameter ≈ 250 μm. After fertilization, the egg nucleus changed its position and forwarded peripherally. The nucleus entered directly into prophase after penetration of the sperm. The egg began to cleave with a surface spiral furrow and maintained the yolk material in the center. The first cleavage furrow was observed 30 minutes post fertilization. The second furrow, which led to stage 4, was introduced from the periphery in the middle between the two separate core districts. Stage 4 thus consisted of four equally large blastomeres and was the first step towards the formation of clear resting cores. The eight blastomeres were approximately equal in size and rounded off. They lied on each other and optimally left a space in the rounding of the inserted germ cells. Cleavage lasted 13 hours and at each cleavage division, blastomeres remained of equal size but increased in number, each instar showed a prominent

nucleus and clear cleavage furrow. Blastulation followed formation of an outer lining of blastoderm surrounding the central yolk material. On the ventral side of the egg, a thickened germinal disk formed that invaginated inward and formed the blastopore. The larval development of *M. japonicas* is like that of other penaeids. The first Nauplius stage hatched and had undergone five successive molts in 36 hours. In all instars, it contained 3 pairs of bristled legs; namely uniramous antenna I, biramous antenna II and a biramous mandible (Figure 1a1 & a2).

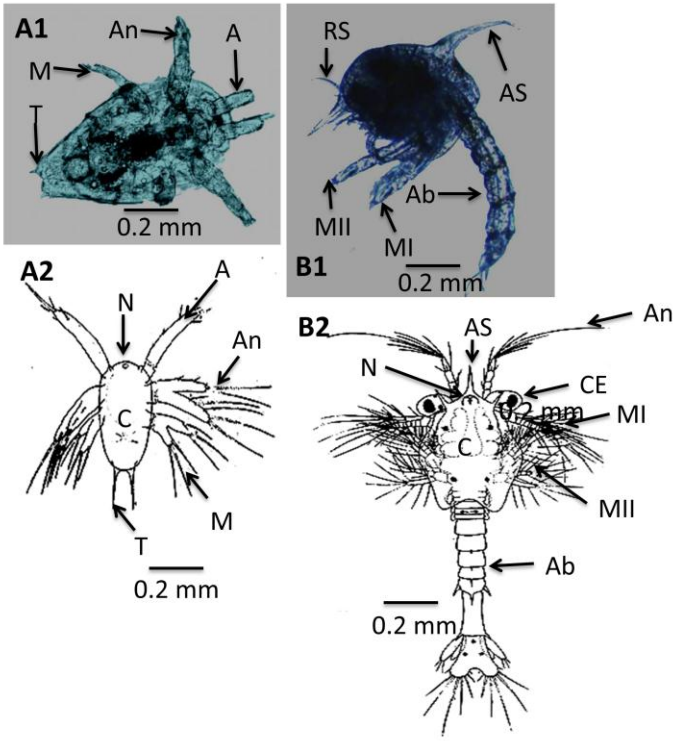


Figure 1. Different larval stages of *M. japonicus*. *M. japonicus* females were artificially inseminated and subsequent larvae were cultured. **A.** nauplii **B.** zoea **C.** mysis **D.** postlarval stage.

A Atennule; An Antenna; M Mandibule; C Carapace; N Nauplius Eye; T Telson; CE Compound Eye; S Spine; Ab Abdomen; MI Maxilliped I; MII Maxilliped II; RS Rostral Spine; CX Cephalothorax; U Uropod; R Rosterum; P Pereiopod; Pl Pleopod

The larval body was broader anteriorly and narrower posteriorly with no segmentation. There was an ocellus in the anterior part of the body. Posteriorly there were two furcated spines. As the larva proceeded in its development, the bristles on its legs increased in number and became longer meanwhile the spines posteriorly became straight. As the larva developed, the posterior part of the body became more tapered and the furcated spines increased. Our study divided the development of Nauplius into initial, early and late stages. The initial stage: Duration: 8 hr 10 min ; Size: Lt 66-78 μ, Ratio cephalothorax / abdomen 0.59. This stage was with pyriform body; furca caudal 1 + 1 of ventral or sub-terminal position; Appendages with smooth bristles,

never feathery or setose; exopodito of the antenna with 5 bristles; reddish nauplii eye. Antenna with 2 apical bristles of similar size, yellowish brown color. Early Nauplius: Duration: 8 hr 40 min; Size: Lt 85-99 μ, Ratio cephalothorax / abdomen 0.75. Furca caudal 3 + 3, due to the addition of 2 pairs of small bristles, an external and internal pair, to the furcal spines. Setose appendages; antenna with 2 terminal feather-like bristles, one long and one medium. Antennal exopodito with 7 processes. Endopodite with 5 processes. The segmentation of the thorax becomes more noticeable. Late Nauplius: Duration: 9hr 10 min; Size: Lt 130-145 μ, Ratio cephalothorax / abdomen 0.85. Body apparently divided into a cephalic region and a thoracic-abdominal region. Anterior cephalic region was with a pair of frontal projections and a median dorsal protuberance. Thoracic-abdominal region was thinner than the cephalic region. Caudal furca was 7 + 7 by aggregation of a pair of internal bristles. Telson was low, with 2 lobes very evident. Ventrally, the labrum or lip and the 4 pairs of appendages posterior to the jaw were noticeably evident. Zoea larval stages were four and lasted 96 hours (Figure 1b1 & b2). this stage has two staked compound and one sessile eyes; (head horizontal, slightly down at the tip; antenna biramous, cephalothorax not segmented, pereiopods 1 and 2 biramous and rudimentary, telson triangular in spatula in form. Our study divided the development of Zoea into early, mid and late stages. During these three stages the body was divided into two main parts: the cephalon or head and the rest of the body composed of the thorax and abdomen (pereion + pleon). The head was covered by a carapace that had a slightly hexagonal shape, being longer than wide, with a maximum width located in the middle of it. The presence of the carapace was a distinctive character between Zoea and Nauplius. The rear part of the carapace had a notch in the middle part and lacks thorns. Another characteristic feature of the stage was the compound eyeswhich became pedunculated. The eye or nauplii eye was maintained and located in the midline, between the compound eyes. The thorax was segmented, presenting between 5-6 segments. The abdomen developed with 5 and 6 segments in the last stage, in addition in the last stage the 6^{th} somite was segmented, leaving the telson free, together with the appearance of the rudimentary uropods. In this stage antennae that fulfill swimming function were well developed, together with maxillipeds 1 and 2. The jaws are incorporated into the buccal region as chewing appendages, having lost the exo and endopodite and with a chewing surface, which it used in its nutrition. The maxillule and maxilla were functional, in addition to the maxillipeds 1 and 2. The 3^{rd} maxilliped and the rudimentary 5 pairs of pereiopods appeared. The telson separated from the 6^{th} abdominal somite, showing a median notch and two lateral lobes that have 8 processes in each lobe. Dorsally, the abdomen or pleon carried a bristle on the posterior border of the 1^{st} to 5^{th} somite and a pair of bristles on the posterior-posterior edge of the 5^{th} and 6^{th} abdominal somites. Mysis larval stages were three and lasted 120 hours (Figure 1c1 & c2). The body was elongated with a prominent cephalothorax and a segmented abdomen. Each segment of the cephalothorax had one pair of legs. A telson appeared between the two swimming legs terminally. As the larva developed, each abdominal segment developed one pair of uniramous unsegmented walking legs. Finally, in the last Mysis stage, each walking leg changed into two segments and thoracic legs became chelated. In the post larval stage, a

rostrum developed with two rostral teeth (Figure 1d1 & d2). The body had lengthened and became more like a small shrimp with specific features more defined. The most notorious and characteristic features that define the stage were the presence of setose exopodites which were well developed in the maxillipeds and pereiopods. Gradual development of the rudimentary pleopods in the abdomen, but without bristles. The cephalothorax presented a pointed apex that projected between the eyes, and a pair of supraorbital bristles of smaller size. The anterolateral edges of the carapace have a series of 5 to 7 spines. The rest of the carapace was smooth. The end of the process of larval development in shrimp is marked by the appearance of the post-larva, which looks very similar to juvenile and adult. The most notorious morphological feature is the presence of a blunt cephalothorax and setose pleopods, as well as minor differences in the shape of the antennule, antenna, maxillipeds, pereiopods, pleopods and telson shape. The shrimp comprised between 12 mm of total length and 65-75 mm of total length is determined as juvenile. The endopodites of the first pair of pleopods grow from the middle of the inner margin of the protopodite of the appendix; when they make their appearance they measure approximately ⅓ of the length of the basipodite. In the juveniles the characteristics of the species are very evident. The number of rostral teeth is not the final one of the adult who reaches 14 and 17. From 30-35 mm unequal growth is observed between both sexes, being greater of the female. The number and percentage of larval transformations under normal and treated conditions with respect to time duration was commented (Table 2). The ratio of cephalothorax length to abdomen length was calculated for Zoeae to young shrimps (Table 3).

Table 2. Number and percentage of larval transformations under normal and treated conditions with respect to time duration

Bioactive inducers	**Conc.**	**after 8hr 10 min laying initial Nauplii**	**after 24 hr 10 min late Nauplii**	**after 50 hr 50 min early Zoea**	**after 96 hr 10 min late Zoea**	**after 120 hr 00min early Mysis**
Sea water	--	59	59	29 49.1	34 57.6	40 76.7
MF	2.5 mM	64	64	53 82.8	57 89.0	61 95.5
	300 µM	60	60	26 43.3	30 50.0	36 60.0
C8	10 µM	67	67	25 37.3	33 49.2	33 49.2
	1 µ M	68	68	49 72.0	59 86.7	65 95.5
	0.1 µM	63	63	29 46.0	35 55.5	39 61.9
ADMA	1 mM	65	65	31 47.6	36 55.3	42 64.6
	1.5 mM	64	64	27 42.1	36 56.2	36 56.2
	2.5 mM	69	69	56 81.1	62 89.8	65 94.2

serotonin	10 µg/ml	63	63	28	49	54
				44.4	77.7	85.7
Acetyl choline	1 mM	63	63	22	32	32
				34.9	50.7	50.7
	0.5 mM	79	79	60	66	66
				75.9	83.5	83.5

Table 3. Measurement of larvae and postlarvae stages of *Marsupenaeus japonicus.*

Stage	cephalothorax length (µm)	abdomen length (µm)	Ratio cephalothorax / abdomen
early Zoea	80.2	80.0	0.99
mid Zoea	84.6	83.1	1.01
late Zoea	90.2	89.6	0.99
early Mysis	98.9	106.3	1.07
mid Mysis	101.2	128.4	1.26
late Mysis	117.5	150.4	1.28
	117.1	186.1	1.58
early post-larva	122.3	233.8	1.91

The effect of pharmacological inducers on larval transformation of *Marsupenaeus japonicus* showed that under treated conditions of MF 2.5 mM, C8:0 1 µM, Asymmetric dimethylarginine (ADMA) 2.5 mM, and Acetyl choline 0.5 mM larval transformation from Nauplius to zoea to early mysis stages was accelerated than larvae of control in sea water (Figure 2a). Serotonin 10 µg/ml and Acetyl choline 1 mM exerted a negative effect while C8:0 0.1 or 10 µM, MF 300 µM, Asymmetric dimethylarginine (ADMA) 1 mM and 1.5 mM did not affect larval transformation.

This study investigated the impact of the lipid-regulating agents Atrovastatin (5, 20 & 40 mg) & Pravastatin (50 mg & 100 mg), the antihistaminic agent Cetrizine hydrochloride (10 mg), the β-blocker Nebivolol (2.5mg, 5 mg. & 10 mg) & Atenelol (25 mg & 100 mg) and the calcium-channel blocker Amlodipine Besylate (2.5mg , 5.0mg , 10mg) on larval transformation from nauplii to early mysis. It was found that these pharmaceuticals exerted a negative impact on larvae in all stages and mortality predominated (Figure 2b).

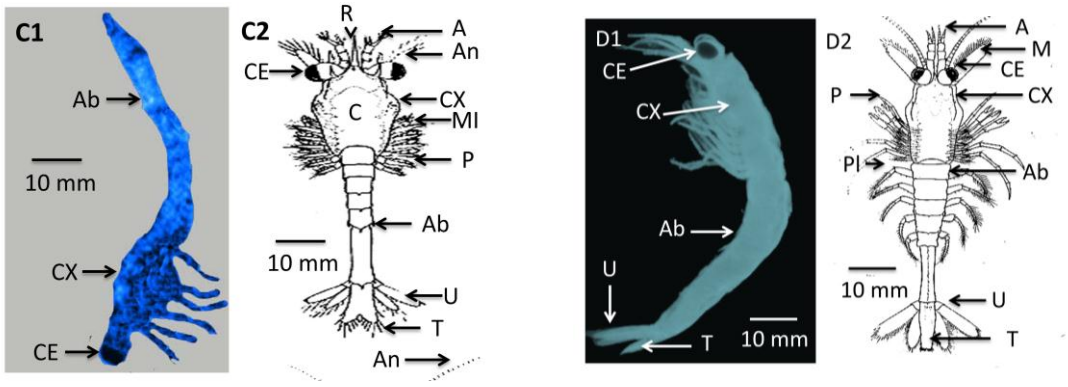


Figure 2. Effect of bioactive compounds on the development of *M. japonicus*. A. Percentage larval transformation in the presence of reagents involved in larval molting and growth, signaling systems and neuronal control (neurotransmitter). **B.** Number of transformed larvae in the presence of the lipid-regulating agents, antihistaminic agents, β-blockers and calcium-channel blockers.

Expression of RF-amide neuropeptide during the different developmental stages

Examination of napluii, zoeae, mysis and postlarval stages of *Marsupenaeus japonicas* showed that RF-amide neuropeptide was mantled in the brain and peripheral neurons of napluii and zoea stages (Figure 3a,b). The RF-amide neuropeptide diffused from the brain and spread in the peripheral neurons of post-larval stages (Figure 3c).

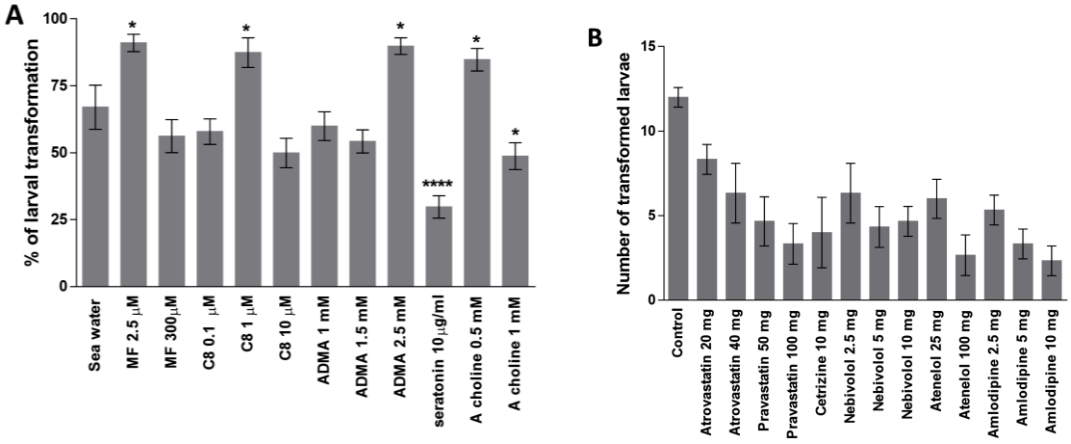
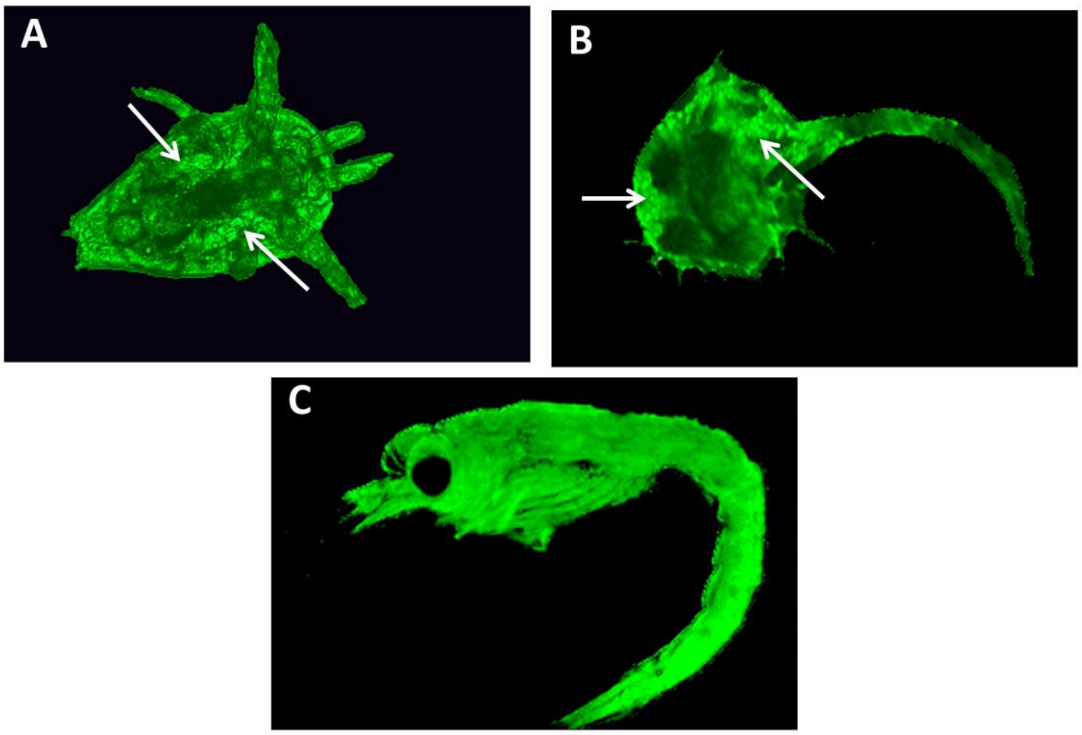


Figure 3. Expression of RF-amide neuropeptide in the different larval stages of *M. japonicus*. Immunohistological staining of RF-amide showed differential expression in the different larval stages. **A.** nauplii **B.** zoea **C.** postlarval stage. Luminescence of RF-amide neuropeptide is indicated by arrows.



DISCUSSION

In any aquaculture system it is necessary to approximate the conditions of captivity to the natural biota of the species (Schulz, et al. 2005). For the success of captive breeding it is also important to know the reproductive biology and larval development of the species in question, through the study and application of techniques suitable for induction of breeding behavior, for hatching and maturation in captivity (Subasinghe, 2001). Perceiving the physical-chemical parameters of water becomes essential, because the water quality, its maintenance, monitoring and its source are necessarily the most important factors for the success of any aquaculture Landau (1992). An organism may be adversely affected when placed in abiotic characteristics other than those indicated for this species, which causes a reduction of their immunity and resistance against certain pathogenic micro-organisms (Wang and Chen, 2004). In Carida prawns the long and variable larval stage is exceptionally delicate (Wunsch, 1996), and species with long larval stages usually have a high mortality (Calado et al., 2003). The larval metamorphosis of *L. amboinensis* was completed by Wunsch (1996) which, despite this, obtained a very high mortality rate (99%). For this species several problems have been reported, including the long larval period and to induce the last molt, when the planktonic Zoea transforms to benthic Post-larva (Simoes, et al. 2003). Thus, one of the largest problems in the aquaculture of Carida prawns is the lack of knowledge of their biology and the great ignorance about the type of feeding and optimal growing conditions. Few species of shrimps have been successfully cultivated in captivity; as a consequence, there is very little information about its larval development, its physiology and ecology, and there is a huge knowledge gap at the their dietary needs (Lin et al., 2001; Rufino and Jones, 2001; Simoes et al., 2003).

In this study we sought to determine the parameters for artificial incrimination in *Marsupenaeus japonicus* and the effect of different compounds on the transformation of larvae in aquaculture. Among the 60 electroejaculation tests performed, we achieved a 63.3% success rate. The percentage of electroejaculation tests, performed in this experiment, was higher than that found by Goldberg & Annibal (1998), who obtained 50% of ejaculations, after application of a single dose of 4.5 V stimuli with freshly harvested *M. rosenbergii* males. The storage of spermatophores in artificial insemination ensures access to the material regardless of availability of males. Chow et al. (1985) successfully obtained larvae of *M. rosenbergii*, from females inseminated with stored spermatophores in liquid nitrogen vapor for 30 days (Chow, et al. 1985). Goldberg & Annibal (1998), through the same technique with the same species, obtained sperm viability of 64.8% after 60 days (Goldberg & Annibal, 1998). Although there are no specific studies on the minimum quantity of sperm needed for insemination of females, Sandifer & Smith (2009) obtained successful fertilization in females of *M. rosenbergii* with only the use of fragments of the ejaculate (Sandifer & Smith, 2009). On the basis of the data obtained by Wickins & Beard (1974) for *M. rosenbergii* (30,000 eggs per spawning and average 80% spawning), the use of spermatophores obtained in the present study in artificial insemination are able to provide insemination of 38 females which would produce 912,000 larvae (Wickins & Beard, 1974).

The present study showed that larvae of *M. japonicus* can be obtained in unlimited quantities by artificial fertilization, and a few hours after heterologous fertilization larvae undergo transformational metamorphosis. It was observed that sperm entry launches contraction waves of the egg cortex leading to specific alterations of the cell shape, which can easily be observed with the microscope. This result coincides with that of Kostyuchenko and Dondua (2000) who showed that cortical contraction components of the cytoplasm of the *Polychaete Nereis virens* become shifted and rearranged in a process termed ooplasmic segregation. In the present study the zygote of *M. japonicus* underwent early determinate cleavages that were radial and symmetric about the midline. The first cleavage furrow divides the embryo into left and right, the second cleavage furrow into anterior and posterior, and the third cleavage furrow into animal hemisphere and vegetal hemisphere. The blastula stage of *M. japonicus* was somewhat spherical in outline and surrounded with the blastoderm. The generous provision of yolk in the egg prevented the appearance of a blastocoele cavity and the surface ornamentation of blastderm was completely different from that of the preceding stages. This observation coincided with the results of other investigators (Berrill, 1947a, 1947b; Cloney, 1969; Grave, 1921; Nishida, 1987). Gastrulation proceeded in accordance with the description of Hertzler & Clark, 1992 who revealed that the gastrula stage of *Sicyonia ingentis* is spherical shaped and the macromeres are condensed with yolk material (Hertzler & Clark, 1992).

The present investigations showed that the nervous system first appeared in the first larval stage as a sensory vesicle and later as the larval stage underwent further development, this vesicle gradually changes into a nervous mass which was hardly differentiated. This observation is in agreement with Conklin (1905) who showed a

cortical region, containing the majority of the neuronal cell bodies and with the larger (10 µm) ones lying closest to the surface, surrounding a mainly fibrous medulla or neuropil (Conklin, 1905). Embryologically, the adult brain arose at a level corresponding to the rostrum (Schulte et al. 1998). The present study showed that the RF amide neuropeptide was present in the neuronal cells of the larvae in all stages and distributed throughout the PNS in all somatic cells. Gradually this protein diminished and disappeared from the neuronal cells in the post larva, but still present in excess in all somatic cells. These observations were in agreement with Bishop et al. (2001) and Bishop and Brandhorst (2001) who reported that inhibition of RF amide neuropeptide function arrested morphogenesis (Bishop et al. 2001; Bishop & Brandhorst, 2001). It can be postulated that the brain secreted neuropeptide signals the rest of the body to stimulate larval molting and transformation.

Several studies have shown the effects of MF (Methyl Farnesoate), 1,2-dioctanyl-rac-glycerol (C8), Serotonin (5-HT), and acetylcholine in signaling systems, neuronal control (neurotransmitters) or control of morphogenetic and behavioral reactions on metamorphosis of marine invertebrates (Leitz & Müller, 1987; Urrutia, et al. 2004; Bishop et al. 2008; Glebov et al. 2014). 5-hydroxytryptamine (5-HT) and serotonin were proven to accelerate metamorphosis in hydroids, ascidians, barnacles and molluscs (McCauley, 1997; Zega, et al. 2005; Glebov et al. 2014). However, our data show that serotonin has a negative effect on the transformation of *Marsupenaeus japonicus* larva. Consistent with our study Glebov et al. (2014) showed that serotonin inhibits development of *Helisoma trivolvis* (Mollusca) in premetamorphic stages (Glebov et al. 2014). Nagaraju & Borst (2008) extended previous studies of the two-color phases of the crab *C. maenas* and provided the first evidence that MF acts as a link between environmental changes and the stimulation of crustacean reproduction (Nagaraju & Borst, 2008). This supports our finding that MF accelerates the transformation of *Marsupenaeus japonicus* larva. 1,2-dioctanyl-rac-glycerol (C8) has been shown to accelerate the rate of metamorphosis in the planula larvae of *H. echinata* consistent with our results in *Marsupenaeus japonicus* (Leitz & Müller, 1987). Finally, it was found that acetylcholine accelerated the larval transformation of *Marsupenaeus japonicus* as shown in previous studies on the short-neck clam *Ruditapes philippinarum* (Urrutia, et al. 2004).

Regulation of metamorphosis in solitary ascidians, sea urchin and a gastropod has been shown to involve Nitric oxide (NO) signaling (Bishop & Brandhorst, 2001). It is now generally recognized that ADMA is a nitric oxide (NO) antagonist. Its principal adverse effect is thought to be uncoupling of the enzyme endothelial nitric oxide synthase (eNOS) (Shibata et al. 2008). Consistent with the previous studies our study showed that ADMA (2.5 mM) in the culture of nauplii enhanced and accelerated larval transformation *Marsupenaeus japonicus*. Therefore, ADMA can be considered as larval metamorphosis factor and is associated with development and growth.

Ali, et al. (2016) studied selected pharmacological compounds, with known mode of action in vertebrates, on the development, metabolism and settlement of larvae of the common fouling barnacle, *Amphibalanus Amphitrite* (Al-Aidaroos, et al. 2017). Nauplii were treated with Atrovastatin, a lipid-regulating compound, cetirizine

hydrochloride, an anti-histamine, atenolol, a beta-blocker, and amlodipine, a calcium-channel blocker. The presence of these compounds delayed the cypris stage when compared with the control. These compounds also inhibited the settlement of cyprids on petri dishes. While exposure to these compounds led to a decrease in the metabolic activity of stage III nauplii, it increased the respiratory rate of cyprids. In this study we also show that lipid-regulating compounds, anti-histamines, beta-blockers and calcium-channel blockers negatively effect larval transformation of *Marsupenaeus japonicus* and increased mortality rates. To avoid larval mortality, the water of the aquaculture tanks must be cleaned regularly from pollutants such as nitrite and all chemical compounds present in the sea water tanks that result from anthropogenic activities and affect directly the general life and embryology of shrimps.

Our results showed that electroejaculation releases significant quantities of spermatophores in *Marsupenaeus japonicus* which can be used in artificial insemination to produce viable larvae. Furthermore, our study showed that specific concentrations of Methyl Farnesoate, 1,2-dioctanyl-rac-glycerol, Acetyl choline and Asymmetric dimethylarginine accelerate larval transformation. Taken together our results establish new methodologies to improve the aquaculture of the economically important shrimp *Marsupenaeus japonicus.*

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Supp. Table 1. Impact of pharmacological inducers on larval transformation of *Marsupenaeus japonicus*

Bioactive inducer	Conc.	No. tested larvae	stage	after 16 hr 40 min Early Nauplius			after 50 hr 50 min Early Zoea			after 80 hr 15 min mid Zoea			after 96 hr 10 min late Zoea			after 120 hrs Late early Mysis		
				1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail
Sea water	--	20* 21** 18***	N Z M D	20	21	18	9 10 - 1	8 9 - 4	8 10 - -	7 12 - 1	7 11 - 4	6 11 - 1	2 16 - 2	5 11 - 5	4 13 - 1	- 12 7 1	1 10 5 5	2 9 6 1
MF	2.5 mM	22* 23** 19***	N Z M D	22	23	19	4 17 - 1	3 20 - -	2 16 - 1	2 18 - 1	1 22 - -	1 17 - 1	- 15 5 1	- 16 7 -	- 10 8 1	- 1 20 1	- - 23 -	- - 18 1

	300 µM	17* 20** 23***	N Z M D	17	20	23	9 7 - 1	10 9 - 1	11 10 - 2	7 9 - 1	8 11 - 2	6 10 - 2	4 11 - 2	5 13 - 2	6 12 - 5	2 11 3 1	3 9 5 3	4 10 5 4
C8	10 µ M	21* 22** 24***	N Z M D	21	22	24	8 7 - 6	10 8 - 4	11 10 - 3	6 9 - 6	7 12 - 4	9 12 - 3	5 9 - 7	6 11 - 5	7 13 - 4	3 8 3 7	1 8 7 6	2 9 8 5
	1 µ M	21* 24** 23***	N Z M D	21	24	23	7 14 - -	5 18 - 1	6 17 - -	2 19 - -	1 21 - 1	4 19 - 1	- 15 6 -	- 18 4 1	- 17 5 1	- 1 20 -	- 1 22 1	- - 23 -
	0.1 µ M	21* 20** 22***	N Z M D	21	20	22	10 10 - 1	10 9 - 1	11 10 - 1	7 13 - 1	9 10 - 1	5 12 - 1	6 13 - 2	7 13 - 2	8 13 - 1	5 4 11 1	5 6 7 2	6 8 7 1
ADMA	1 mM	21* 24** 20***	N Z M D	21	24	20	11 10 - -	10 12 - 2	11 9 - -	9 11 - 1	8 13 - 3	9 10 - 1	7 13 - 1	5 16 - 3	6 13 - 1	4 8 6 1	3 10 9 3	5 12 2 1
	1.5 mM	19* 20** 25***	N Z M D	19	20	25	8 7 - 4	11 9 - -	12 11 - 2	7 10 - 4	10 13 - 2	9 14 - 2	5 9 - 5	7 12 - 1	8 14 - 3	3 8 2 6	5 6 7 2	4 10 7 4
	2.5 mM	21* 22** 26***	N Z M D	21	22	26	5 16 - -	4 17 - 1	2 23 - 1	2 19 - -	2 19 - 1	1 24 - 1	- 14 6 1	- 13 7 2	- 16 9 1	- 1 19 1	- - 21 1	- - 25 1
serotonin	10 µg/ml	20* 22** 21***	N Z M D	20	22	21	10 10 - -	10 11 - 1	14 7 - -	10 10 - -	12 9 - 1	12 8 - 1	9 10 - 1	11 8 - **2**	10 9 - 2	- 9 10 1	1 11 8 3	2 10 7 2
Acetyl choline	1 mM	22* 21** 20***	N Z M D	22	21	20	13 7 - 2	12 8 - 1	12 7 - 1	10 10 - 2	9 11 - 1	8 11 - 1	8 10 - 4	9 10 - 2	10 9 - 1	5 11 1 5	6 10 3 2	6 9 3 2
	0.5 mM	23* 26** 28***	N Z M D	23	26	28	3 19 - 1	5 21 - -	7 20 - 1	2 20 - 1	3 23 - -	4 23 - 1	1 15 6 1	- 15 10 1	1 11 15 1	- - 21 2	- 1 25 -	- - 27 1

* = No. of tested larvae in the first trial N = Nauplius larva Z = Zoea larval
** = No. of tested larvae in the second trial M = Mysis larva D = dead larva at any stage
*** = No. of tested larvae in the third trial ;

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