Controlled reproduction of penaeid shrimp: a contribution to its improvement

Jorge Alfaro Montoya

Promotoren: prof.dr. E. A. Huisman
Hoogleraar in de Visteelt en Visserij

Co-promotoren: Dr. J. Komen
Universitair docent, leerstoelgroep Visteelt en Visserij

Samenstelling promotiecommissie:

Prof.dr.. H.J.Th. Goos (Universiteit Utrecht)
Prof.dr. D. van der Heide (Wageningen Universiteit)
Dr. J.H.W.M. Rombout (Wageningen Universiteit)
Dr. C. Cahu (Unité Mixte de Nutrition des Poissons
IFREMER-INRA, Frankrijk

Controlled reproduction of penaeid shrimp: a contribution to its improvement

Jorge Alfaro Montoya

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
prof.dr. ir. L. Speelman
in het openbaar te verdedigen
op 18 April 2001
des namiddags te vier uur in de Aula

Table of contents

Stellingen

Belonging to the thesis

"Controlled reproduction of penaeid shrimp: a contribution to its improvement"

Jorge Alfaro Montoya

Wageningen, 18 April 2001

1. Injections, with 17-alpha-methyltestosterone improve quality of shrimp spermatophores.
   This thesis.

2. Serotonin injections induce ovarian maturation in shrimp, be it at a lower rate than unilateral eyestalk ablation.
   This thesis.

3. Late stages of shrimp embryos have a better tolerance to cooling than early stages of bovine embryos.
   This thesis and Wilmut et al. (1975), The effect on cow embryos of cooling to 20, 0 and –196 °C. J. Reprod. Fertil. 45, 409 – 411.

4. The pituitary gland in fish and the brain/ thoracic ganglia in shrimp have similar functions in the endocrine control of reproduction.

5. Genetic selection of shrimp for stress tolerance will improve the quality of gametes and therefore, larvae production.

6. Scientific advances in shrimp mariculture are contributing to the recovery of over-exploited wild populations.

7. Humanity is increasingly accumulating scientific knowledge. So, there is scope for understanding God's creation.

8. To reach the moon, one must have breakfast first.

For my daughter, Valeria

Chapter 1

General introduction

Scope of the thesis

Marine shrimp aquaculture has become a major industry since the pioneering works in larvae culture and pond culture by M. Fujinaga (Shigueno, 1975), eyestalk ablation in a penaeid shrimp (Caillouet, 1972), and closing the life cycle in captivity of some shrimp species (Aquacop, 1979).

Global production of farmed shrimp has grown at 8% annually since 1989. In 1998, the production reached 1,113,887 mt, representing around 32% of world shrimp and prawn catches, based on FAO fishery statistics (www.fao.org) and FAO (2000). The industry has expanded mostly in Southeast Asia and Latin America, where different penaeid species are cultured. The most important species in Latin America is *Penaeus (Litopenaeus) vannamei*, followed by *P. (Litopenaeus) stylirostris* and *P. (Litopenaeus) schmitii*. In Asia *P. monodon*, *P. chinensis*, *P. (Marsupenaeus) japonicus*, *P. penicillatus*, *P. merguiensis*, and *P. indicus* are cultured (Weider and Rosenberry, 1992).

In Asia, shrimp culture started back in 1962 in Japan. By 1991 Southeast Asia contributed 80.6% of the global shrimp production; the leading producers were China, Indonesia and India. In 1997, the total production of captured and cultured shrimps / prawns from Asia was estimated at 2.95 million mt. Cultured shrimp represented around 18% of this total yield, based on FAO fishery statistics. Current leading producers are Thailand, India and Indonesia (Weidner and Rosenberry, 1992; Anonymous, 1999). Thailand has been the world leading producer since 1992, with an estimated production of 243,800 mt in 1998 (FAO, 2000).

In Latin America, shrimp farming started around 1962 in Ecuador. During the 1980 decade, other Latin American countries initiated shrimp farming activities, reaching or surpassing in few years their own shrimp catches (Colombia, Honduras; Weidner and Rosenberry, 1992). In 1991 the contribution to the global production was 19.4%, and the leading producers were Ecuador, Colombia, and Mexico (Weidner and Rosenberry, 1992). In 1997 fisheries and aquaculture in Latin America generated around 0.63 million mt, with shrimp farming representing 34% of this production (FAO fishery statistics; Anonymous, 1999). By 1998 the contribution of Latin American countries to the global shrimp production had increased to 29%, with Ecuador as the major producer and the second largest in the world with 144,000 mt (Anonymous, 1999; FAO, 2000).

In recent years, commercial shrimp operations have experienced dramatic losses due to disease outbreaks, caused by viruses, bacteria, fungi, and parasites (Brock and LeaMaster, 1992; Lightner, 1992; Wang et al., 1998; Itami et al., 1998; Morales-Covarrubias and Chavez-Sanchez, 1999). Viral diseases especially have had a major impact on shrimp production worldwide. In 1988-89 Taiwan experienced serious

economic losses due to Monodon-type baculoviruses and its production decreased to extremely low yields (4,812 mt of *P. monodon* in 1998; FAO, 2000). A few years later *Baculovirus penaei* appeared in America and the infectious hypodermal and hematopoietic necrosis virus (IHHNV) dispersed in Southeast Asia and America (Lightner, 1992). In 1992 a new virus appeared in the Taura region from Ecuador, the Taura syndrome virus (TSV), which caused severe economic losses throughout America (Hasson et al., 1999). In the same year the white spot syndrome virus (WSSV), which has a wide host range and infects various decapods at varying infectivity rates (Wang et al., 1998), affected Southeast Asia. As a result production in Southeast Asia decreased to 71% of the world shrimp production (Anonymous, 1999). WSSV has been recently (1999) identified in Ecuador and Central America, and low productions are expected. However, American countries may learn from the Asian experience (Jory, 2000).

Shrimp farming is based on wild and cultured postlarvae, depending on regional characteristics for postlarvae availability. Reproduction of marine shrimp is now performed in controlled environments, where wild or cultured animals mature and release their gametes. This technological approach allows a predictable production of nauplius larvae. In the past only wild animals were utilized, but nowadays pond-grown and selected animals are becoming more important as spawners because, as survivors from viral diseases, they might present genetic resistance to the specific virus.

The traditional dependency on wild stocks for nauplii production will thus change towards a more controlled production of genetically improved animals, selected for important aquaculture traits like growth rate and disease resistance (Hedgecock and Malecha, 1991). However, the genetic improvement of shrimp requires the application of breeding programs, which relay on excellent gamete quality, controlled fertilization, and, if possible, cryogenic storage. For this a proper understanding of basic shrimp reproductive biology, and the development of *in vitro* fertilization techniques and cryogenic protocols for gametes, embryos, and larvae are needed.

Female maturation is achieved by unilateral eyestalk ablation. This technique is applied to each female at intermolt, selecting one of the eyestalks. Few days after the procedure, ovaries will start to grow and first spawnings will occur within a week, with an increasing rate in the subsequent days. Each female generates various spawns, but eventually the spawning activity decreases, until around 2 to 3 months after ablation, when they are replaced by new females. Eyestalk ablation affects all aspects of shrimp physiology (Quackenbush, 1986) and over time it has a deleterious effect on spawn quality and quantity, as well as on survival (Emmerson, 1980; Primavera, 1985; Tsukimura and Kamemoto, 1991; Benzie, 1998). This dependency on eyestalk ablation

is a major bottleneck for the advance of controlled reproduction, and major research activities are dedicated to develop hormonal treatments for controlled maturation.

Traditionally, penaeid shrimp males have received less attention than females, assuming that they will always give high quality sperm. While this may be so for males caught from the wild, it is not the case for pond-reared animals nor for males from the wild after a few months in captivity. Male shrimp also show reproductive problems (Brown et al., 1979; Chamberlain and Gervais, 1984), they are often affected by infections of the reproductive tissue, including ampoules, vas deferens, and testis; the condition renders males infertile. Chamberlain and Lawrence (1981) stated that the reproductive capabilities of the males may be a limiting factor in captivity, indicating that a better understanding of spermatophore production and analysis of sperm quality is important in shrimp mariculture (Leung-Trujillo and Lawrence, 1987).

In vitro fertilization is essential for selective breeding programs as it allows controlled mating designs, but it has not been developed yet. Some data have been published on this subject, giving contradictory results on fertilization rates and indicating that our understanding of the fertilization mechanism in open and closed thelycum shrimp is still fragmentary. Clark et al. (1973) reported 10% fertilization for *P aztecus*, Alfaro et al. (1993) got no fertilization for *P. occidentalis*, Misamore and Browdy (1997) obtained 2.48% for *P. setiferus* and 3.88% for *P. vannamei*. A 50% hatching rate was reported for *P. monodon* (Lin and Ting 1984 cited in Primavera, 1985).

Nauplii are shipped in plastic bags with chilled (20 °C) and oxygenated seawater at 15,000 to 25,000 nauplii l^{-1} (Kungvankij et al., 1986). International trading as well as breeding programs would be positively influenced by cryopreservation of penaeid shrimp seedstock (Anchordoguy et al., 1988; Benzie, 1998), but these techniques have not yet been developed (Benzie, 1998; Subramoniam and Arun, 1999).

The aim of the present thesis was to address some of the problems in shrimp reproduction as discussed before. Various penaeid species were selected for conducting the research (Table 1). This selection of species was based on several criteria such as commercial interest, ready availability, and ability to spawn in captivity. First, the reproduction of *Penaeus occidentalis* in Gulf of Nicoya, Costa Rica, and an *in vitro* fertilization protocol using good quality gametes from wild broodstock, were evaluated. The quality of spermatophores, addressing both pathological and culture effects, was studied in 3 penaeid species. To improve spermatophore quality the effect of an androgen and a progestagen was evaluated. Serotonin treatment was evaluated in females as an alternative to eyestalk ablation for ovarian maturation and spawning.

Table 1. Characteristics of the penaeid species selected for the studies of this thesis.

Penaeid species	Characteristics		
	Thelycum	Habitat	Reason for selection
Penaeus occidentalis	Open	Gulf of Nicoya	Model/ Fisheries
Penaeus vannamei	Open	Gulf of Panamá/ Farms	Aquaculture
Penaeus stylirostris	Open	Farms	Aquaculture
Penaeus setiferus	Open	Gulf of Mexico	Model
Trachypenaeus byrdi	Closed	Gulf of Nicoya	Model

Finally, embryo tolerance to cooling, cryoprotectants, and hypersaline exposure was assessed as a first step towards defining cryogenic protocols.

In the next section of this chapter, the sexual characteristics of shrimp are presented. The section on "reproductive endocrinology" describes the present understanding of the hormonal pathways involved in reproduction of decapod crustaceans. The section on "controlled reproduction of shrimp" defines the specific aspects and environmental variables required for controlled reproduction of shrimp. Finally, the last section gives an overview and an integration of the studies performed for this thesis.

Reproductive biology of penaeid shrimp

Primary and secondary sexual characteristics.

Penaeid shrimp are broadcast decapod-spawners (Dendrobranchiata). The general structure of the female reproductive system consists of a pair of multi-lobed ovaries (Fig. 1), located in the dorsal region over the stomach, the hepatopancreas, and the intestine. The oviducts are simple and open to the exterior at the coxae of the third pair of walking legs (King, 1948). Oocyte maturation is divided in two phases, primary vitellogenesis and secondary vitellogenesis (Adiyodi and Subramoniam, 1983; Charniaux-Cotton, 1985). Primary oocytes store glycoproteins produced by themselves, then follicle cells proliferate around each oocyte and secondary vitellogenesis is activated, accumulating vitelline produced in hepatopancreas, hemocytes and ovaries (Yano and Chinzei, 1987; Quackenbush and Keely, 1988; Quackenbush, 1989).

Marine shrimp females of the family Penaeidae show two distinctive modifications of the sternal plates located between third, fourth and fifth walking legs (Perez-Farfante, 1975; Browdy, 1992). This region is known as the thelycum, and it may present ornamentations for spermatophore attachment on the day of spawning (**open** thelycum shrimp like *P. vannamei*, *P. stylirostris*, *P. setiferus*, and *P. occidentalis*), or a seminal receptacle for sperm storage (**closed** thelycum shrimp like *P.*

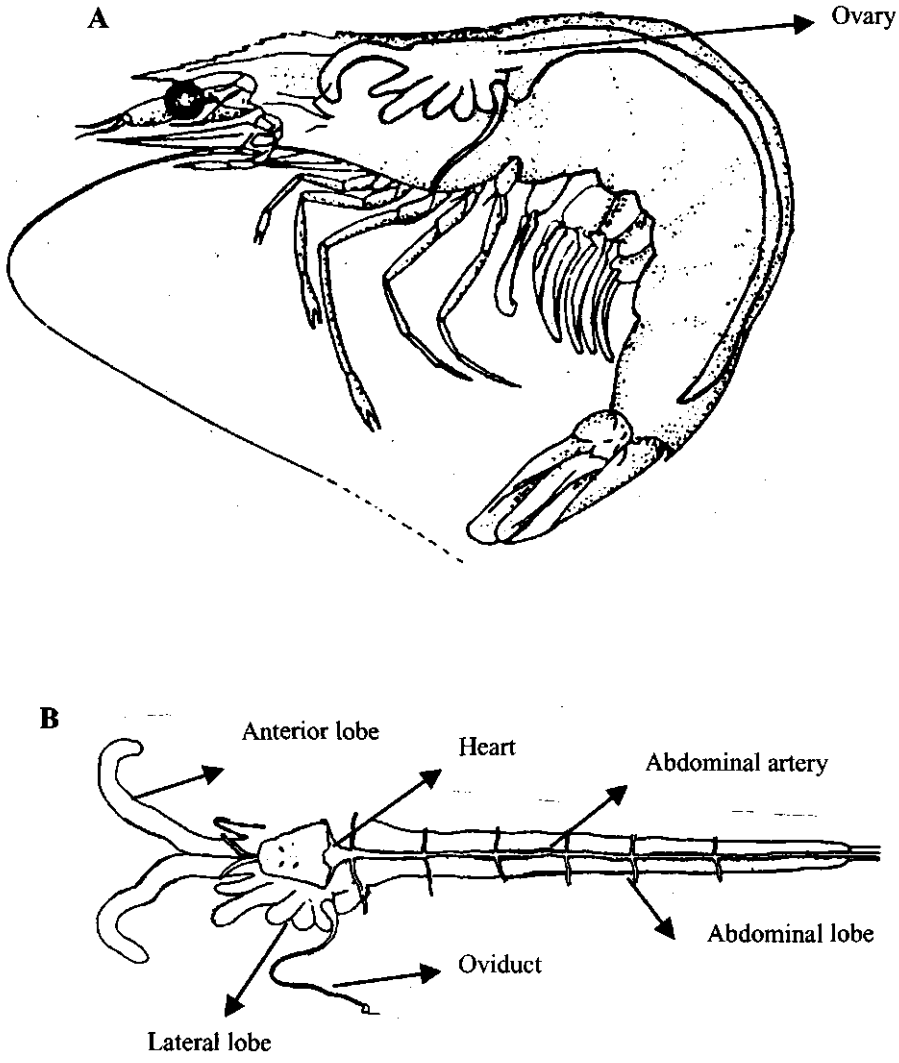

Figure 1. Female reproductive system of penaeid shrimp. A: Anatomical location of the reproductive system. B: Isolated reproductive system (modified from Shigueno, 1975).

aztecus, *P. monodon*, and *P. japonicus*).

Males are recognized by the presence of a modification of exopodites of the first pair of swimming legs (pleopods). The modification consists of a membranous development named petasma, which seems to participate in spermatophore transfer and attachment. The male reproductive system presents a pair of dorsal multi-lobed testis (Fig. 2). The spermiducts or vas deferens are very complex, showing the following sections (starting from the testis): blind pouch next to the testis, ascending medial vas deferens, descending medial vas deferens, distal duct, and terminal ampoule, which opens at the coxae of the fifth pair of walking legs (pereiopods, Talbot et al., 1989). Two distinctive channels are observed inside the medial vas deferens.

Spermatogenesis in the penaeid shrimp, *Sicyonia ingentis*, was extensively studied at the ultrastructural level by Shigekawa and Clark (1986). In the testis, sperm cells are immature, and they complete maturation in the vas deferens (open thelycum species) or in the seminal receptacles (closed thelycum species). Sperm cells do not have flagella, instead they have a non-motile "spike" (Clark et al., 1984; Shigekawa and Clark, 1986). A particularly interesting subject is spike elongation, proved to be a gradual process in *P. stylirostris* that takes place in the descending medial vas deferens (Fig. 3). *T. byrdi* males present spikeless sperm even in tissue sections from the distal vas deferens and ampoules (Fig. 3; Alfaro, 1994), but light microscopical observations of sperm from spermatophores reveal fully elongated spikes (Alfaro, unpublished data). Therefore, as it was pointed out by Shigekawa and Clark (1986) for *S. ingentis*, *T. byrdi* sperm experiences rapid elongation that inevitably must take place within the spermatophores. Recent light microscopical observations in *T. byrdi* show that sperm morphology in spermatophores removed from males and in spermatophores removed from female seminal receptacles is similar. In both cases, sperm are packed in many 160 μm-capsules (spermatophores), the spike is deflected 80° from the body axis, the sperm body length is 7 – 9 μm, and the spike length 4 μm. These particular characteristics have also been observed in *Xiphopenaeus riveti*, which has very large sperm: a body length of 13 μm with a spike length of 6.5 μm (Alfaro, unpublished data). In *S. ingentis*, sperm are capacitated by the female's thelycum, where further development within the sperm cells takes place (Clark et al., 1984; Shigekawa and Clark, 1986).

Spermatophore primary and secondary layers are synthesized in the ascending and descending vas deferens, respectively (Ro et al., 1990). The main body and sperm sac of the spermatophore originate in one of the two channels of the vas deferens (Bauer and Cash, 1991). The terminal ampoule is histologically complex, showing separated chambers where additional spermatophore sub-units (dorsal plate, wings, glutinous mass, adhesive) are formed in open thelycum species (Talbot et al., 1989).

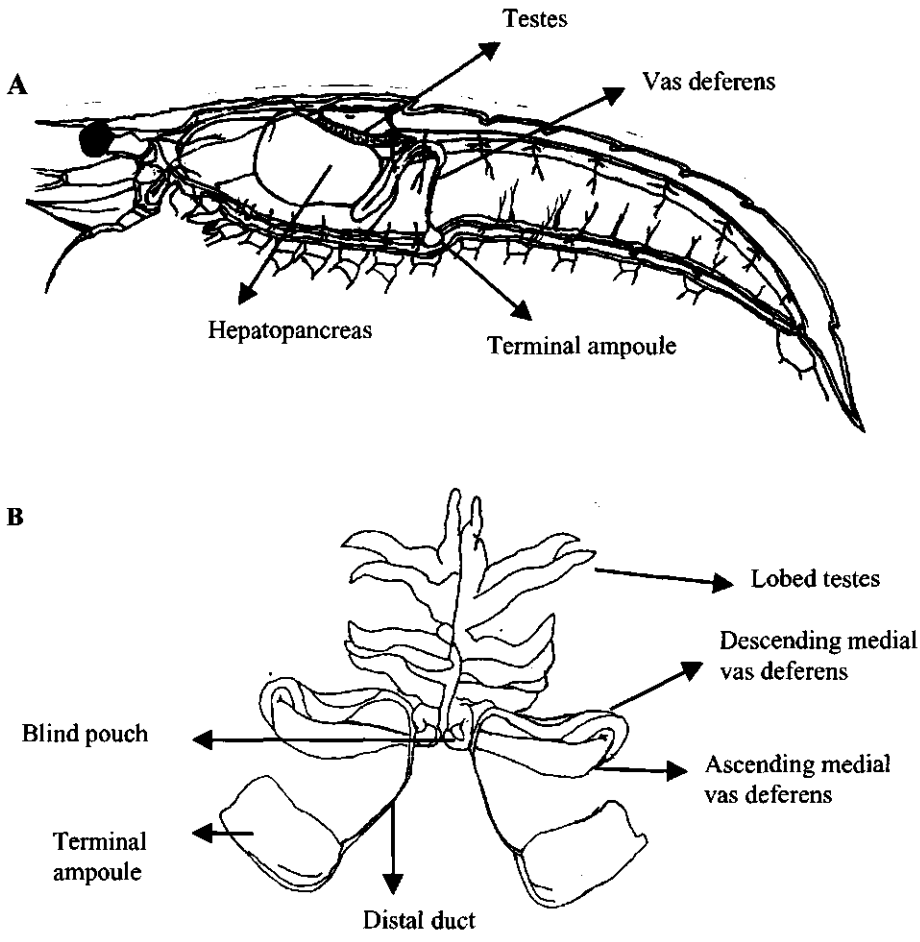

Figure 2. Male reproductive system of penaeid shrimp. A: Anatomical location of the reproductive system (modified from Shigueno, 1975). B: Isolated reproductive system (modified from King, 1948).

Figure 3. A) *Trachypenaeus byrdi* spermatid from the distal vas deferens. The nucleus presents fibrillar chromatin (CF) and membrane lamellar bodies (CLM). Between the anterior granule (GA), from the acrosome, and the granular core (CG), from the subacrosome, a new subacrosomal region develops, the saucer plate (P). B) *Penaeus stylirostris* spermatids from the descending medial vas deferens. Spikes with different degrees of elongation (From Alfaro, 1994).

Penaeids with closed thelycum have lost the adhesive and glutinous materials, and the dorsal plate functions as a stopper to plug the opening of the thelycum (Chow et al., 1990; Bauer and Cash, 1991).

Reproductive endocrinology

There are 6 endocrine centers believed to be directly involved in reproduction of decapod crustaceans. The first one is the ganglionic medulla terminalis - X organ – sinus gland complex from the optic ganglia. Other centers are the brain, the thoracic ganglia, the androgenic gland (males), the mandibular organ, and the ovaries (Fig.4). No endocrine activity has been identified in the testis.

Crustaceans with pedunculated eyes have a group of neurosecretory cells in the eyestalks, the X organ. These cells send the majority of their axons to a neurohemal organ, the sinus gland (Quackenbush, 1986). The medulla terminalis – X organ synthesizes polypeptides, that are packed in neurosecretory vesicles and transported intra-axonally to the sinus gland, where they are stored and released as small peptides (Andrew, 1983). The whole complex is involved in the regulation of physiological processes like calcium and sugar metabolism, heart rate, reproduction, molting, osmoregulation, thermal seasonal acclimation, retinal pigments migration, and color change (Quackenbush, 1986).

The mechanism proposed for the control of gonadal maturation is an antagonistic model (Fig.4), which involves the synthesis of a gonad inhibiting hormone (GIH) from the X organ. This peptide was recently isolated and sequenced from a lobster and a crayfish (Huberman, 2000). The hormone inhibits or competes with a hypothetical gonad stimulating hormone (GSH), produced in the brain and thoracic ganglia. The GSH activates secondary vitellogenesis in females, and spermatogenesis, hypertrophy of vas deferens and androgenic gland hypersecretion in males (Adiyodi and Subramoniam, 1983). GSH is an abstract entity, but its release is stimulated by 5-hydroxytryptamine and the red pigment-concentrating hormone; dopamine and methionine enkephalin inhibit GSH release, and stimulate GIH release (Fingerman, 1997).

The androgenic gland is the endocrine organ, which determines primary and secondary male sexual characteristics (Charniaux-Cotton, 1960; Fingerman, 1987; Charniaux-Cotton and Payen, 1988). Two androgenic gland hormones, AGH 1 and AGH 2, were isolated from an isopod by Hasegawa et al. (1987). Other androgenic gland extracts like farnesylacetone and steroids have been identified in decapods, and they may play complementary roles to AGH (Sagi, 1988).

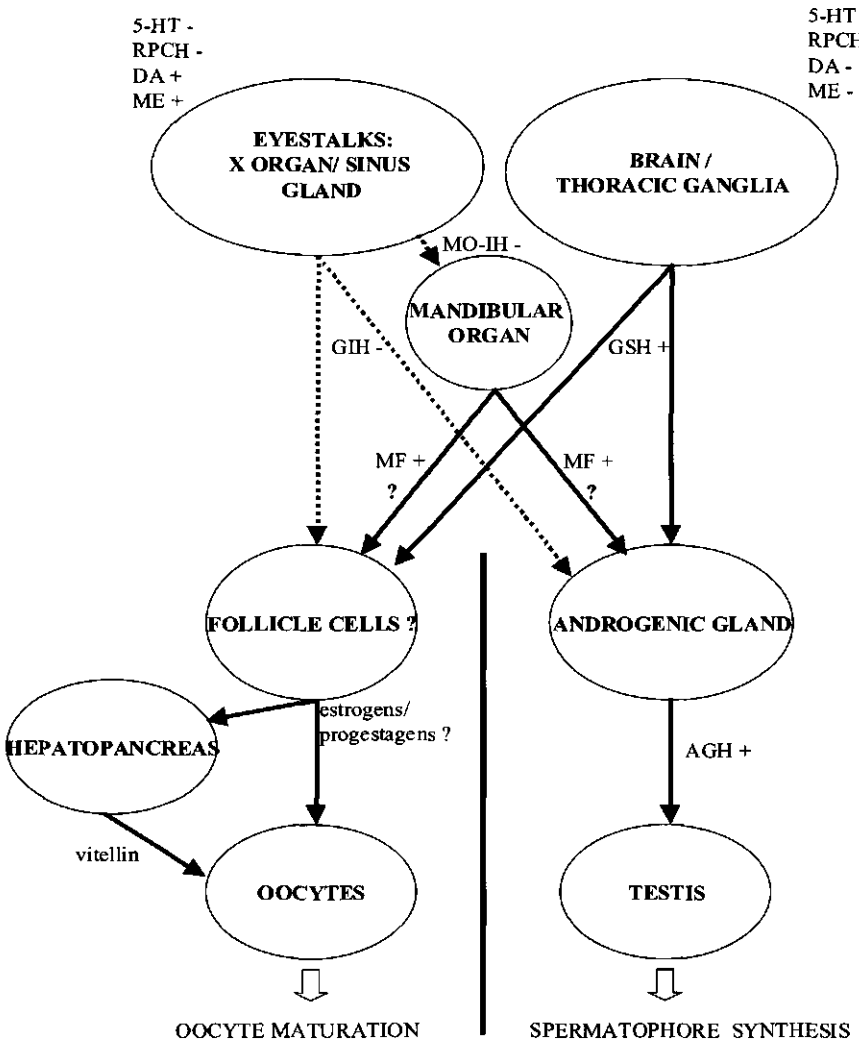

Figure 4. Proposed pathways in endocrine control of reproduction in decapod crustaceans. Continuous lines indicate a stimulatory effect; dotted lines indicate an inhibitory effect; question marks indicate unconfirmed pathways. 5-HT: serotonin, RPCH: red pigment-concentrating hormone, DA: dopamine, ME: methionine enkephalin, GSH: gonad stimulating hormone, GIH: gonad inhibiting hormone, MO-IH: mandibular organ inhibiting hormone, MF: methyl farnesoate, AGH: androgenic gland hormone. The model is based on the antagonistic effect of GSH and GIH over the gonads. GSH induces oocyte maturation, presumably through steroid hormones. The mandibular organ also stimulates oocyte maturation.

It has been proposed that ovarian development and oocyte maturation in crustaceans may be mediated by steroid hormones, as in fish and amphibia, where estrogens stimulate vitellogenesis and progestagens induce oocyte maturation (Fairs et al., 1990). In the lobster, *Homarus americanus,* these hormones are believed to be produced in the follicle cells (Talbot, 1981).

The mandibular organ located near the esophagus, produces steroids and terpenoids, that stimulate ovarian development (Tsukimura and Kamemoto, 1991). The terpenoid, methyl farnesoate (MF), was identified by Laufer et al. (1987). Its synthesis is increased by unilateral eyestalk ablation, and the compound is considered a reproductive hormone as it induces ovarian maturation in the spider crab, *Libinia emarginata* (Que-Tae et al., 1999). A mandibular organ inhibiting hormone from the X organ has been partially identified (Huberman, 2000, for review).

The first invertebrate neuropeptides biochemically characterized were the red pigment-concentrating hormone and the distal retinal pigment light adapting hormone of crustaceans (Josefsson, 1983). Other crustacean hormones recently characterized are the molting hormone (20-OH-ecdisone; Chang, 1985), the molt-inhibiting hormone (neuropeptide), and the hyperglycemic hormone (neuropeptide) (Benzie, 1998; Huberman, 2000, for review).

Controlled reproduction of shrimp

In Latin America wild or pond-grown *P. vannamei* and *P. stylirostris* are matured in captivity. Resistant shrimp are selected from the cultured population based on growth performance, kept in earthen ponds at a low density (1 m^{-2}), and fed a combination of artificial feeds and fresh marine food products. However, at present avoidance of fishery products is recommended as a preventive measure against WSSV in America (Jory and Dixon, 1999). Animals from ponds are grown to 45 g for females and 40 g for males, and then transferred to a maturation facility.

Males are stocked with females at a 1:1 ratio in large tanks (> 3.7 m in diameter), under low light intensity, high water exchange (> 100% daily), and avoiding any kind of stress. To mature females in controlled environments, it is necessary to feed marine animals like fish, oysters, worms, clams, and squids. These products will give females an adequate level of essential nutrients: amino acids, cholesterol, and polyunsaturated fatty acids (PUFAs). Oocyte maturation and survival of initial larval stages are associated with the accumulation within oocytes of sterols (synthesized from dietary cholesterol), phospholipids, triacylglycerols, and diacylglycerols (synthesized from dietary PUFAs; Ravid et al., 1999). Embryo quality is affected by stored nutrients so that acceptable hatching rates and nauplii quality are obtained when broodstock is fed products from the marine food chain. Worms are particularly rich in PUFAs,

including 20:5w3 and 22:6w3, which are considered as crucial nutrients for crustacean maturation (Harrison, 1990).

Seawater used for maturation must imitate the natural habitat, which is normally oceanic for penaeid reproduction. However, some species may reproduce in estuarine waters such as *P. occidentalis* (Alfaro et al., 1993). Generally, salinity is maintained between 28 and 36 ppt, temperature in the range of 27 to 29 °C, pH from 8.0 to 8.2, dissolved oxygen over 5 ppm, NH_4-N below 0.1 mg l^{-1}, and NO_2-N below 0.05 mg l^{-1} (Bray and Lawrence, 1992). To maintain a high quality environment, water is exchanged at rates of over 100% daily, in flow-through systems.

Reproduction of marine shrimp can be accomplished using a broad variety of light sources and intensities. In general terms, it is adequate to use natural or artificial cool white light at low intensity (< 5 $\mu Em^{-2}s^{-1}$), with gradual increase and decrease of light level, providing 14 to 16 hours of light per day (Chamberlain, 1988; Bray and Lawrence, 1992). Mating under laboratory conditions requires adequate space (tanks > 3.7 m in diameter) as well as reduced turbulence and noise to facilitate courtship and success of spermatophore transfer, and handling should be minimized (Browdy, 1998).

When females have completed oocyte maturation, they will be receptive to males to coordinate the mating behavior and spermatophore transfer, particularly at dusk. Insemination may occur under natural mating or using artificial insemination. A few hours after spermatophore transfer, spawning takes place and sperm-egg interaction initiates at the moment of water contact. Eggs show a cortical reaction, characterized by the release of cortical rods containing jelly precursors and sperm activators; at the same time, sperm undergoes acrosomal reaction, leading to a secondary binding to the ovum's oolema (Clark et al., 1984). Eventually, germinal vesicle breakdown occurs and pro-nuclei are fused. A hatching envelope is formed by the release of two different types of cortical vesicles: dense vesicles and ring vesicles (Pillai and Clark, 1988). Embryonal development in penaeids leads to the formation of a hollow blastula, which evolves into the larval stage named nauplius. The nauplius emerges approximately 14 hours (28 °C) after spawning.

Commercial facilities for nauplii production are integrated with maturation facilities. Impregnated and ripe females are individually transferred to spawning tanks (100 - 200 l), where eggs develop their embryonal stages on the bottom of the tank. High quality water (1 µm filtration and U.V. disinfection) must be used (McVey and Fox, 1983) and egg density should not exceed 3000 eggs l^{-1} to get acceptable hatching rates (Primavera, 1985).

Eggs with hatching envelope or nauplii are rinsed with clean seawater for 5 min, concentrated in a bucket, disinfected with formalin (eggs: 100 ppm/ 1 min; nauplii: 400

ppm/ 30 sec) and iodine (0.1 ppm/ 1 min), and washed with clean seawater for 5 min (Browdy, 1992).

Outline of this thesis

The aim of this thesis was to provide new information for a better understanding of penaeid reproductive biology, and to investigate some of the problems associated with controlled reproduction of shrimp. The steps followed in this thesis are schematized in Fig. 5.

First we explored the natural events involved in *P. occidentalis* reproduction in a tropical estuary, the Gulf of Nicoya, Costa Rica, where this species is the predominant penaeid. This study also investigated a protocol for *in vitro* fertilization, using high quality gametes, as an alternative to natural fertilization (**Chapter 2**). The next step was to investigate spermatophore quality in penaeids. In captivity, male shrimp develop reproductive problems. The pathology of a disease of the reproductive system, which was not understood earlier, was studied in a susceptible species, *P. setiferus* (**Chapter 3**). Spermatophore production in pond-grown males was studied in *P. stylirostris* (**Chapter 4**). Additionally, quality and natural deterioration of spermatophores was further investigated in pond-grown *P. vannamei* (**Chapter 5**). Next, the research was focused on finding a hormonal treatment for the improvement of male sexual condition (**Chapter 6**). Based on the current knowledge about crustacean endocrinology, 17 alpha-methyltestosterone and 17 alpha-hydroxyprogesterone were evaluated. Ovarian maturation and spawning are currently induced through unilateral eyestalk ablation. The effect of serotonin treatment on female maturation was investigated in wild *P. vannamei*, as a less invasive alternative (**Chapter 7**). A final step in this research process was to get a knowledge base for future embryo and nauplii cryopreservation. In doing so, **Chapter 8** explores embryo and nauplii sensitivity to cooling, cryoprotectants and hypersaline exposure, using a wild species easy to spawn in captivity, *T. byrdi*. In **Chapter 9** the overall results are analyzed, and an integral interpretation of these results is discussed.

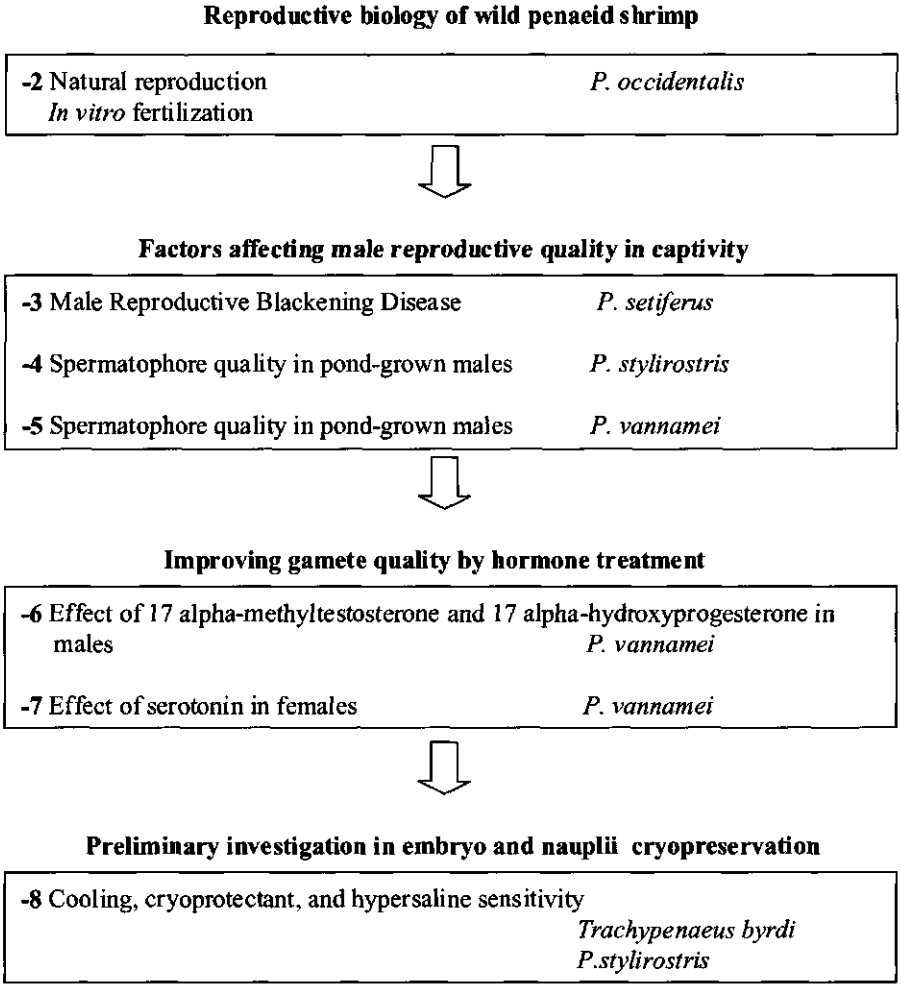

Figure 5. Schematic representation of the research steps followed in this thesis. Numbers indicate specific chapters addressing the subject.

References

Adiyodi, R. G., Subramoniam, T., 1983. Arthropoda – Crustacea (oogenesis, oviposition and oosorption). In: Adiyodi, K. G. (Ed.), Reproductive biology of crustacea, vol. 1: oogenesis, oviposition and oosorption, John Wiley and Sons Ltd., London, pp. 443 – 495.

Alfaro, J., 1994. Ultraestructura de la glándula androgénica, espermatogénesis y oogénesis de camarones marinos (Decapoda: Penaeidae). Rev. Biol. Trop., 42, 121 – 129.

Alfaro, J., Palacios, J. A., Aldave, T. M., Angulo, R. A., 1993. Reproducción del camarón *Penaeus occidentalis* (Decapoda: Penaeidae) en el Golfo de Nicoya, Costa Rica. Rev. Biol. Trop. 41, 563 – 572.

Anchordoguy, T., Crowe, J. H., Griffin, F. J., Clark, W. H. Jr., 1988. Cryopreservation of sperm from the marine shrimp *Sicyonia ingentis*. Cryobiology 25, 238 – 243.

Andrew, D., 1983. Neurosecretory pathways supplying the neurohemal organs in crustacea. In: Gupta, A. P. (Ed.), Neurohemal organs of arthropods: their development, evolution, and functions, Charles C. Thomas, Springfield, IL, USA, pp. 90 – 117.

Anonymous, 1999. A review of world shrimp farming. Aquaculture Magazine Buyer's Guide'99 and Industry Directory. 28th Annual Edition. Aquaculture Magazine, Asheville, NC, USA, pp. 40 – 58.

Aquacop, 1979. Penaeid reared broodstock: closing the cycle of *P. monodon*, *P. stylirostris* and *P. vannamei*. Proc. World Maric. Soc. 10, 445 – 452.

Bauer, R. T., Cash, C. E., 1991. Spermatophore structure and anatomy of the ejaculatory duct in *Penaeus setiferus*, *P. duorarum*, and *P. aztecus* (Crustacea: Decapoda): homologies and functional significance. Trans. Am. Microsc. Soc. 110 (2), 144 – 162.

Benzie, J. A. H., 1998. Penaeid genetics and biotechnology. Aquaculture 164, 23 – 47.

Bray, W. A., Lawrence, A. L., 1992. Reproduction of *Penaeus* species in captivity. In: Fast, A., Lester, L. J. (Eds.), Marine shrimp culture: principles and practices, Elsevier Science Publishers B. V., Amsterdam, The Netherlands, pp. 93 – 169.

Brock, J. A., LeaMaster, B., 1992. A look at the principal bacterial, fungal and parasitic diseases of farmed shrimp. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 212 – 226.

Browdy, C. L., 1992. A review of the reproductive biology of *Penaeus* species: Perspectives on controlled shrimp maturation systems for high quality nauplii production. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 22-51.

Browdy, C. L., 1998. Recent developments in penaeid broodstock and seed production technologies: improving the outlook for superior captive stocks. Aquaculture 164, 3 – 21.

Brown, A., Jr., McVey, J. P., Middleditch, B. S., Lawrence, A. L., 1979. Maturation of the white shrimp (*Penaeus setiferus*) in captivity. Proc. World Maric. Soc. 10, 435 – 444.

Caillouet, A. C. Jr., 1972. Ovarian maturation induced by eyestalk ablation in pink shrimp, *Penaeus duorarum* Burkenroad. Proc. World Maric. Soc. 3, 205 – 225.

Chamberlain, G. W., 1988. Stepwise investigation of environmental and nutritional requirements for reproduction of penaeid shrimp. PhD Thesis, Department of Wildlife and Fisheries Sciences, Texas A&M University, USA.

Chamberlain, G. W., Lawrence, A. L., 1981. Effect of light intensity and male and female eyestalk ablation on reproduction of *Penaeus stylirostris* and *P. vannamei*. J. World Maric. Soc. 12, 357 – 372.

Chamberlain, G. W., Gervais, N. F., 1984. Comparison of unilateral eyestalk ablation with environmental control for ovarian maturation of *Penaeus stylirostris*. J. World Maric. Soc. 15, 29 – 30.

Chang, E. S., 1985. Hormonal control of molting in decapod crustacea. Amer. Zool. 25, 179 – 185.

Charniaux-Cotton, H., 1960. Sex determination. In: Waterman, T. H. (Ed.), The physiology of crustacea, vol. 1 Metabolism and growth. Academic Press, New York, New York, USA, pp. 411 - 447.

Charniaux-Cotton, H., 1985. Vitellogenesis and its control in malacostracan crustacea. Amer. Zool. 25, 197 – 206.

Charniaux-Cotton, H., Payen, G., 1988. Crustacean reproduction. In: Downer, R. G. H. and Laufer, H. (Eds.), Invertebrate endocrinology, vol. 2, Alan R. Liss, Inc., New York, USA, pp. 279 – 303.

Clark, W. H. Jr., Talbot, P., Neal, R. A., Mock, C. R., Salser, B. R., 1973. *In vitro* fertilization with non – motile spermatozoa of the brown shrimp *Penaeus aztecus*. Mar. Biol. 22, 353 – 354.

Clark, W. H. Jr., Yudin, A. I., Griffin, F. J., Shigekawa, K., 1984. The control of gamete activation and fertilization in the marine penaeidae, *Sicyonia ingentis*. In: Engels, W. (Ed.), Advances in invertebrate reproduction 3, Elsevier, New York, USA, pp. 459 – 472.

Chow, S., Sandifer, P. A., Dougherty, M. M., Dougherty, W. J., 1990. Spermatophore formation in penaeid shrimps. In: Hirano, R., Hanyu, I. (Eds.), The Second Asian Fisheries Forum, Asian Fisheries Society, Manila, Philippines, pp. 623 – 626.

Emmerson, W. D., 1980. Induced maturation of prawn *Penaeus indicus*. Mar. Ecol. Prog. Ser. 2, 121 – 131.

Fairs, N. J., Quinlan, P. T., Goad, L. J., 1990. Changes in ovarian unconjugated and conjugated steroid titers during vitellogenesis in *Penaeus monodon*. Aquaculture, 89: 83-99.

FAO, 2000. Aquaculture production 1998. FAO Fisheries Series No. 56. Food and Agriculture Organization. Rome, 169 p.

Fingerman, M., 1987. The endocrine mechanisms of crustaceans. J. Crustacean Biol. 7, 1 - 24.

Fingerman, M., 1997. Roles of neurotransmitters in regulating reproductive hormone release and gonadal maturation in decapod crustaceans. Invertebr. Reprod. Dev. 31, 47-54.

Harrison, K. E., 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: a review. J. Shellfish Res. 9(1), 1 – 28.

Hasegawa, Y., Haino-Fukushima, K., Katakura, Y., 1987. Isolation and properties of androgenic gland hormone from the terrestrial isopod, *Armadillidium vulgare*. Gen. Comp. Endocrin. 67, 101 - 110.

Hasson, K. W., Lightner, D., Mohney, L., Redman, R., Poulos, B., White, B., 1999. Taura syndrome virus (TSV) lesion development and the disease cycle in the Pacific white shrimp *Penaeus vannamei*. Dis. Aquat. Org. 36, 81 – 93.

Hedgecock, D., Malecha, S., 1991. Prospects for the application of biotechnology to the development and improvement of shrimp and prawns. In: Sandifer, P. (Ed.), Advances in World Aquaculture, vol. 4: Shrimp culture in North America and the Caribbean, The World Aquaculture Society, Baton Rouge, Louisiana, pp. 161 – 200.

Huberman, A., 2000. Shrimp endocrinology. A review. Aquaculture 191, 191 – 208.

Itami, T., Asano, M., Tokushige, K., Kubono, K., Nakagawa, A., Takeno, N., Nishimura, H., Maeda, M., Kondo, M., Takahashi, Y., 1998. Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. Aquaculture 164, 277 – 288.

Jory, D. E., 2000. Status of shrimp aquaculture 2000. Aquaculture Magazine Bayer's Guide 2000 and Industry Directory. 29th Annual Edition. Aquaculture Magazine, Asheville, NC, USA, pp. 49 – 60.

Jory, D. E., Dixon, H. M., 1999. Shrimp whitespot virus in the western hemisphere. Aquaculture Magazine 25(3), 83 – 91.

Josefsson, L., 1983. Chemical properties and physiological actions of crustacean chromatophorotropins. Amer. Zool. 23, 507 – 515.

Khin-Maung, O., Kurokura, H., Iwano, T., Okamoto, K., Kado, R., Hino, A., 1998. Cryopreservation of nauplius larvae of the barnacle, *Balanus amphitrite* Darwin. Fish. Sci. 64 (6), 857 – 860.

King, J. E., 1948. A study of the reproductive organs of the common marine shrimp, *Penaeus setiferus* (Linnaeus). Biol. Bull. 94, 244 - 262.

Kungvankij, P., Tiro, L. B., Pudadera, B. J., Jr., Potestas, I. O., Corre, K. G., Borlongan, E., Talean, G. A., Bustilo, L. F., Tech, E. T., Unggui, A., Chua, T. E., 1986. Shrimp hatchery design, operation and management. Network of Aquaculture Centers in Asia, Philippines, 88 pp.

Laufer, H., Borst, D., Baker, F. C., Carrasco, C., Sinkus, M., Reuter, C. C., Tsai, L. W., Schooley, D. A., 1987. Identification of a juvenile-like compound in a crustacean. Science 235, 202 – 205.

Leung-Trujillo, J. R., Lawrence, A., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65, 363 – 370.

Lightner, D. V., 1992. Shrimp virus diseases: diagnosis, distribution and management. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 238 – 253.

McVey, J., Fox, J., 1983. Hatchery techniques for penaeid shrimp utilized by Texas A&M – NMFS Galveston Laboratory program. In: CRC Handbook of mariculture, vol. 1, CRC Press, Inc., Boca Raton, FL, USA, pp. 129 – 154.

Misamore, M., Browdy, C., 1997. Evaluating hybridization potential between *Penaeus setiferus* and *Penaeus vannamei* through natural mating, artificial insemination and *in vitro* fertilization. Aquaculture 150, 1 – 10.

Morales-Covarrubias, M., Chavez-Sanchez, C., 1999. Histopathological studies on wild broodstock of white shrimp *Penaeus vannamei* in the Platanitos area, adjacent to San Blas, Nayarit, Mexico. J. World Aquacult. Soc. 30 (2), 192 – 200.

Perez Farfante, I., 1975. Spermatophores and thelyca of the american white shrimps, genus *Penaeus*, subgenus *Litopenaeus*. Fish. Bull. 73(3), 463 – 486.

Pillai, M. C., Clark, W. H., Jr., 1988. Hatching envelope formation in shrimp (*Sicyonia ingentis*) ova: origin and sequential exocytosis of cortical vesicles. Tissue Cell 20 (6), 941 – 952.

Primavera, H.J., 1985. A review of maturation and reproduction in closed thelycum penaeids. In: Taki, Y., Primavera, J. H., and Llobrera, J. A. (Eds.), Proc. 1st Int. Conf. on the Culture of Penaeid Prawns/Shrimp, Aquaculture Department, SEAFDEC, Iloilo, Philippines, pp. 47-64.

Quackenbush, L.S., 1986. Crustacean endocrinology; a review. Can. J. Fish. Aquat. Sci. 43, 2271-2282.

Quackenbush, L. S., 1989. Vitellogenesis in the shrimp, *Penaeus vannamei*: *in vitro* studies of the isolated hepatopancreas and ovary. Com. Biochem. Physiol. 94 (B), 253 – 261.

Quackenbush, L. S., Keely, L. L., 1988. Regulation of vitellogenesis in the fiddler crab, *Uca pugilator*. Biol. Bull. 175, 321 – 331.

Que-Tae, J., Laufer, H., Biggers, W., Sung, H., 1999. Methyl farnesoate induced ovarian maturation in the spider crab, *Libinia emarginata*. Invertebr. Reprod. Dev. 36, 79 – 85.

Rabid, T., Tietz, A., Khayat, M., Boehm, E., Michelis, R., Lubzens, E., 1999. Lipid accumulation in the ovaries of a marine shrimp *Penaeus semisulcatus* (De Haan). J. Exp. Biol. 202, 1819 – 1829.

Ro, S., Talbot, P., Leung-Trujillo, J., Lawrence, A. L., 1990. Structure and function of the vas deferens in the shrimp *Penaeus setiferus*: segments 1 – 3. J. Crustacean Biol. 10, 455 – 468.

Sagi, A., 1988. The androgenic gland in crustacea - with emphasis on the cultured freshwater prawn *Macrobrachium rosenbergii* - a review. Isr. J. Aquacult. (Bamidgeh) 40 (4), 107 - 112.

Shigekawa, K., Clark, W. H. Jr., 1986. Spermatogenesis in the marine shrimp, *Sicyonia ingentis*. Dev. Growth Differ. 28, 95 – 112.

Shigueno, K., 1975. Shrimp culture in Japan. Association for International Promotion, Tokyo, Japan, 153 p.

Subramoniam, T., Arun, R., 1999. Cryopreservation and aquaculture: a case study with penaeid shrimp larvae. Curr. Sci. 76 (3), 361 – 368.

Talbot, P., 1981. The ovary of the lobster *Homarus americanus*: structure of the mature follicle and origin of the chorion. J. Ultrastruct. Res. 76, 249 – 262.

Talbot, P., Howard, D., Leung-Trujillo, J., Lee, T. W., Li, W. Y., Ro, H., Lawrence, A. L., 1989. Characterization of male reproductive tract degenerative syndrome in captive penaeid shrimp (*Penaeus setiferus*). Aquaculture 78, 365 – 377.

Tsukimura, B., Kamemoto, F.I., 1991. *In vitro* stimulation of oocytes by presumptive mandibular organ secretions in the shrimp, *Penaeus vannamei*. Aquaculture 92, 59 - 66.

Wang, Y. C., Lo, C. F., Chang, P. S., Kou, G. H., 1998. Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquaculture 164, 221 – 231.

Weidner, D., Rosenberry, B., 1992. World shrimp farming. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 1 - 21.

Wyban, J., (Editor), 1992. Proceedings of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, 301 p.

Yano, I., Chinzei, Y., 1987. Ovary is the site of vitellogenin synthesis in kuruma prawn, *Penaeus japonicus*. Com. Biochem. Physiol. 86 (B), 213 – 218.

Chapter 2

Reproduction of the Shrimp *Penaeus occidentalis* (Decapoda: Penaeidae) in Gulf of Nicoya, Costa Rica

J. Alfaro, J. A. Palacios, T. A. Aldave, R. A. Angulo

Escuela de Ciencias Biológicas, Universidad Nacional,
Heredia, Costa Rica

Published in: Rev. Biol. Trop. (1993), 41 (3):563 - 572
(The General introduction presents schematic figures of reproductive systems; therefore, original photographs were removed from this article)

Abstract

Reproduction of the shrimp *Penaeus occidentalis* was studied between June 26 and July 27, 1992 near Curazao beach, Chira Island, Gulf of Nicoya, Costa Rica. This is the first documentation of reproduction of a penaeid shrimp in Gulf of Nicoya. Spawning behavior was divided in five stages based on these observations; a sperm-egg interaction was evaluated using the *in vitro* spawning technique. Spawned eggs presented a cortical reaction during the first 30 min after release, and the sperm experienced primary binding to the egg vitelline envelope, but an acrosome reaction was not activated. There appear to be differences in fertilization mechanisms between open and closed thelycum penaeid shrimp. Wild *P. occidentalis* males presented heavy spermatophores compared to other open thelycum shrimps. Sperm count (49.52 million per compound spermatophore), and sperm abnormalities (22.0 %), were similar to those of wild *P. setiferus* from the Gulf of Mexico.

Key-words: Shrimp, *Penaeus*, reproduction, spawning, maturation

Introduction

The Gulf of Nicoya is a tropical estuary and has been divided in two sections of different characteristics, the upper gulf and the lower gulf (DeVries et al., 1983). The upper gulf is shallow (< 20 m) with muddy sediments and is bounded mostly by mangrove swamps; the transition between both areas is localized near San Lucas island and Puntarenas peninsula. Some physical-chemical aspects (Peterson, 1969; Valdés et al., 1987), crustacean biology (DeVries et al., 1983; Epifanio and Dittel, 1984) and shrimp fisheries (Carranza, 1985; Vitola, 1985; Palacios et al., 1992) have also been evaluated in the Gulf of Nicoya.

Decapod crustaceans belonging to Penaeidae family are organized in four subfamilies: Aristaeinae, Solenocerinae, Sicyoninae, and Penaeinae. The first two include deep water species and the last two subfamilies include coastal shrimp (Imai, 1980).

Boschi (1979) describes the reproductive cycle of the genus *Penaeus* that consists of two phases. In the first phase, pre-adults migrate from estuarine waters to a reproduction zone in deeper and higher salinity waters. Once the spawning occurs, postlarvae migrate to estuarine waters. This pattern is followed by various species: *P. duorarum*, *P. brasiliensis*, *P. aztecus*, *P. schmitti*, *P. stylirostris*, *P. vannamei*, *P. setiferus* and *P. occidentalis* (Garcia and LeReste, 1981).

The quality of wild spermatophores during natural mating has been partially documented. This type of analysis has been reported for males under culture conditions (controlled reproduction) and wild *P. setiferus* broodstock (Leung-Trujillo and

Lawrence, 1985; Leung-Trujillo and Lawrence, 1987; Chamberlain, 1988; Alfaro, 1990).

Observations about spawning behavior, cortical reaction and sperm-egg interaction have been partially evaluated in penaeid shrimp (Pillai et al., 1988; Clark et al., 1984). Most of our knowledge about fertilization mechanisms in marine shrimp comes from *Sicyonia ingentis* (Sicyoninae) and *P. aztecus* (Clark et al., 1980; Clark et al., 1981; Lynn and Clark, 1987; Pillai and Clark, 1987; Pillai and Clark, 1988; Griffin et al., 1988). Following contact with seawater, penaeid eggs experience a cortical reaction, characterized by a massive release of cortical crypts or rods. Then cortical vesicles are released, which give origin to the hatching envelope. During sperm-egg interaction sperm bind to eggs, and molecules released by the eggs activate the sperm; sperm undergo an acrosomal reaction, characterized by spike depolymerization, and the release of the acrosomal vesicle content. These events are a prerequisite for fertilization.

The spawning behavior has been described for closed thelycum shrimp: *P. japonicus* (Hudinaga, 1942), *P. monodon* (Motoh, 1981) and *S. ingentis* (Pillai et al., 1988). However, in open thelycum shrimp (*P. vannamei*, *P. stylirostris*, *P. occidentalis*), the spawning behavior has not been studied in detail.

This work presents results about maturation and mating in the white shrimp, *P. occidentalis*, from Curazao beach, Chira Island, and observations on their spawning behavior, cortical reaction, and sperm-egg interaction.

Material and methods

Captures

On June 26, 1992, a zone with maturation and mating activity for the species *P. occidentalis* was found. The reproduction zone is localized near Curazao beach, on the northeast side of Chira Island (Fig. 1), at a depth of 2-6 m.

Six fishing trips were assigned to the reproduction zone. Once a week, a monofilament fishing net (mesh size = 7.60 cm) of 212 m long and 2.43 m wide, was cast during a period of 20 min. This procedure was repeated three times. Captures were performed during afternoon hours and the degree of maturation and mating was registered until the end of the closed fishing season (July 31). In addition to field observations, samples were preserved on ice for laboratory analysis.

Maturation and mating

Female maturation was evaluated using an arbitrary scale based on our observations about the degree of development and the coloration of ovaries:

1. Undefined: ovary outline is not distinguishable.

2. Maturing: anterior, lateral, and abdominal ovarian lobules are clearly visible and their coloration is opaque.
3. Almost mature: lobules are highly developed with a yellow to light red coloration.
4. Mature: lobules are turgid with a dark red coloration.

Natural mating was evaluated based on the presence of complete spermatophores, spermatophore residues, or sperm mass, attached to female thelycum.

Controlled spawning and spermatophore analysis

During the fishing trips mature females with and without attached spermatophores, and males with spermatophores were selected. These animals were transported in isolated containers (7 animals/ container) with chilled (23.5 °C) and oxygenated seawater to the Experimental Maturation Laboratory at Criadero de Camarones de Chomes S.A, where experiments on spawning and semen analysis were performed.

In the laboratory, the presence of spermatophores or residues on the thelycum was again evaluated and animals were gradually acclimated to the culture water (temperature = 27 °C, salinity = 35 ppt). Impregnated females were isolated in plastic tanks (40 L) with seawater treated by filtration (silica sand and 1 μm cartridge) and sedimentation. EDTA was added as a chelator at a rate of 10 mg l^{-1} (Chamberlain and Lawrence, 1983) and aeration was provided. By taking 3,0 ml samples (4 replicates), the number of spawned eggs was estimated. Also most of the impregnated females completely removed their sperm mass and spermatophore residues, during the 3 h transportation time. Eight impregnated females and seven mature but not impregnated females were selected for spawning. They were transferred to spawning containers and kept under constant observation until their spawning.

The events associated with the sperm–egg interaction were studied through *in vitro* spawnings using the following two techniques: a) plastic cones, and b) beakers. At the moment of spawning females were captured and held over a 300 ml plastic cone or 100 ml beaker. These containers were previously inoculated with a sperm suspension, obtained by homogenization of two sources: a) sperm mass removed from its spermatophore or medial vas deferens, and b) complete spermatophores. Sperm density in spawning units was estimated by hemacytometer counting (Alfaro, 1990; Alfaro, 1992). To ensure adequate sperm and eggs mixing, plastic cones were provided with an aeration line, and suspensions in beakers were mixed with a goiter. Additionally, ova in beakers were gently swirled, following the protocol for *in vitro* spawning of *S. ingentis* (Griffin et al., 1988).

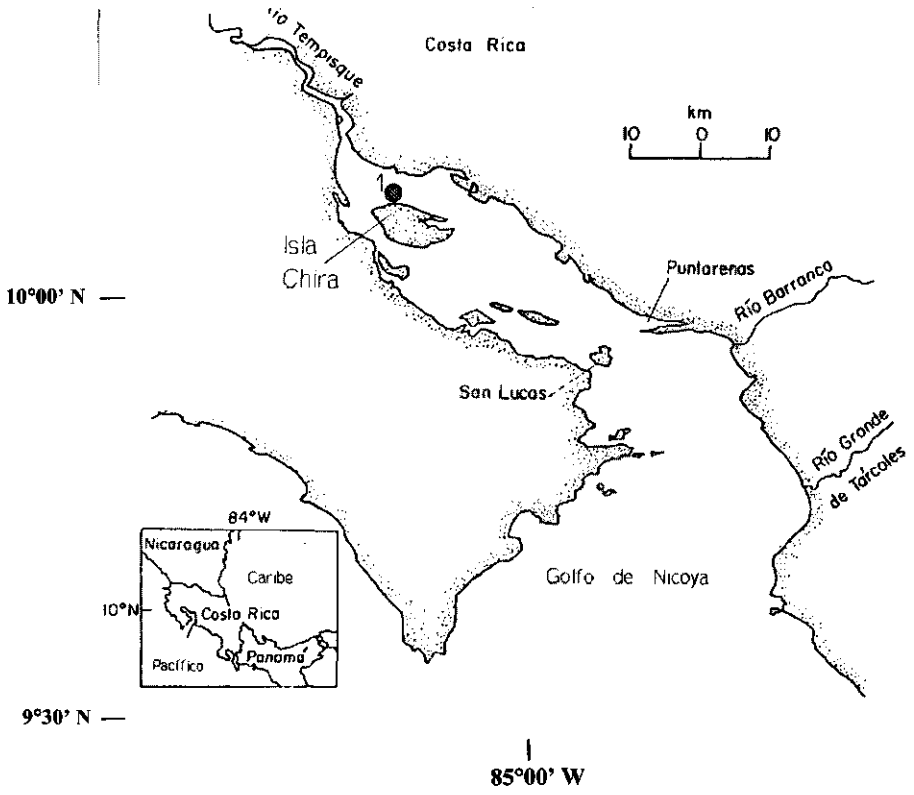

Figure 1. Location of the study area (station 1) in the northeast coast of Chira island, Gulf of Nicoya, Costa Rica.

Sperm analysis involved total sperm count per compound spermatophore (two sub-units), compound spermatophore weight, and percentage of sperm with missing spikes or malformed bodies, following the techniques previously described (Alfaro, 1990; Alfaro, 1992).

Results

The July 7 capture could not be analyzed quantitatively (Figs. 2 and 3). These figures show the frequency of females and males (Fig. 2), ovarian maturation, and rate of mature and impregnated females (Fig. 3) in the reproduction zone.

Capture analysis indicates that during four weeks of consecutive sampling, there were adult males and females in the area. Females were found in stages 1, 2, 3 and 4 of ovarian maturation. It was found that over 40.7 % of mature females captured each week were impregnated. On July 22, one female and 3 males were captured, and on July 27 maturing females appeared again.

Under laboratory conditions, *P. occidentalis* females showed the following spawning pattern:

A) Resting. Females show minimum or no activity, resting on the bottom of the tank. This phase lasted 5-6 hours.

B) Ascension and descension. Females swim to the surface and rapidly return to the bottom. This behavior is repeated, lasting 1 h.

C) Swimming. Females are suspended in the water column, swimming slowly around the tank; pleopods are vigorously agitated and the abdomen is curved. This phase may be short or be repeated some times.

D) Egg releasing. Females release their eggs while showing behavior (C). Eggs descend and are dispersed by the water current generated by pleopods. The ovary content is evacuated partially or completely; a complete spawn takes a few minutes.

E) Spawned. Females rest on the bottom, showing minimum activity. Ovaries have lost their dimension, outline, and color. In partial spawns, females present abdominal ovarian lobes.

Spawning experiments showed that all impregnated and 5 non-impregnated females spawned at dawn the day after their capture. Only 2 impregnated females had abnormal spawns, characterized by the release of oocytes that form masses that stick to the bottom of the tank. Females (mean weight = 63.4 ± 8.7 g) released 26600 to 186600 eggs per spawn.

A particular aspect of this species was that the majority of impregnated females completely removed their sperm mass during transport, possibly as a stress result. For

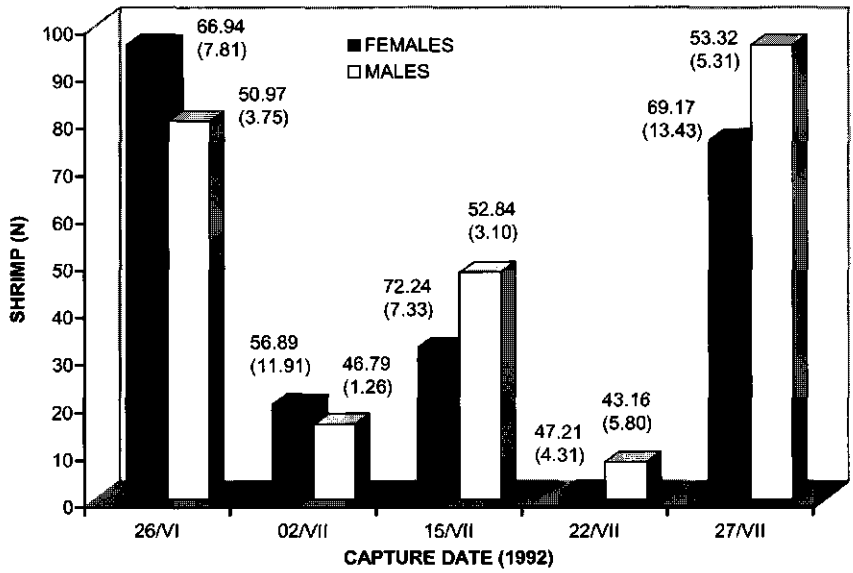

Figure 2. Number of shrimp and average total weight (g) of *Penaeus occidentalis* collected during the study in Chira Island (northwest coast), Gulf of Nicoya. Values in parenthesis represent standard deviation of weight.

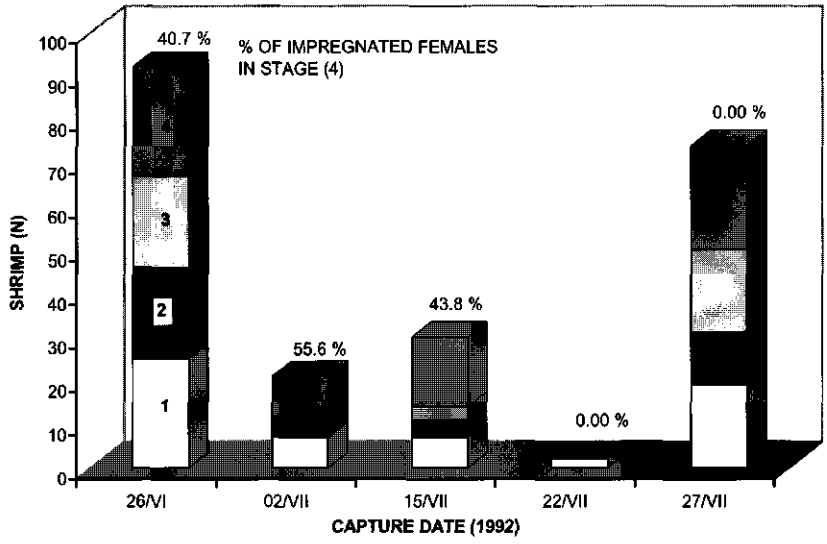

Figure 3. Maturation stages (1, 2, 3 and 4) and rate of impregnated *Penaeus occidentalis* captured in the north-east coast of Chira Island, Gulf of Nicoya.

example, at the moment of capture one female was removing the sperm mass with her claws. Probably, females that arrived to the laboratory with sperm masses, removed them during the night because spawns were infertile. Fertilization in penaeids is external and it occurs at the moment of spawning.

The technique of *in vitro* spawning allowed the evaluation of the cortical reaction of eggs and sperm condition. Table 1 summarizes the cortical reaction events observed in fresh samples. The release of cortical crypts is a massive process that forms a jelly coat of 660 µm in diameter, around the eggs (220 µm). Egg diameter is reduced

Table 1. Cortical reaction events for *Penaeus occidentalis* eggs during *in vitro* spawnings[a]

Time (min)	Event[b]
0	Spawning. Egg diameter = 280 µm.
8	Eggs surrounded by cortical rods.
15	Rod dissipation and hatching envelope formation.
30	Hatching envelope completely developed.

[a] Temperature = 27 °C, salinity = 35 ppt.

[b] The sequence of events occurred in similar way with and without sperm.

during the cortical reaction and this ends with the formation of a hatching envelope; these events do not need the presence of sperm. *In vitro* spawning was practiced by two techniques. Table 2 shows the conditions and observations obtained in the spawnings; no acrosomal reaction of sperm was observed under either technique. Table 3 presents the quality parameters of spermatophores from wild males during a natural period of maturation and mating.

Discussion

This work documents a massive reproductive activity of the species *P. occidentalis* near Curazao beach, Chira island. The evidence indicates that in the region *P. occidentalis* develop ovaries and mate with the imminent release of eggs in the same area. This process was observed between June 26 and July 15, 1992 (closed season for shrimp fishery); samples from July 22 indicate an absence of adults in the area, and the following week (July 27), the activity appears again.

Carranza (1985) established that the upper Gulf of Nicoya is not a reproduction region for white shrimps. However, this study demonstrates that the reproductive cycle of *P. occidentalis* takes place in estuarine and shallow waters. The absence of gravid females in Carranza's study may be the result of selected sampling stations.

Table 2. Observations on *Penaeus occidentalis* sperm in interaction with eggs, during *in vitro* spawnings.

Spawning technique	No. of spawn	Sperm density (cel./ml)[a]	Sperm morphology 0	15	30min
Cone	1	150000[b]	N.C.	N.C.	N.C.
	2	305000[b]	N.C.	N.C.	N.C.
Beaker	3	185000[c]	N.C.	N.C.	N.C.
	4	185000[c]	N.C.	N.C.	N.C.

[a] Sperm suspensions were prepared 1 or 2 hours before spawning.

[b] Sperm removed from spermatophores and medial vas deferens.

[c] Sperm from spermatophores.

N.C. = no change in morphology

Table 3. Spermatophore quality in wild *Penaeus occidentalis*.

Animal	Total weight	Spermatophore weight (g)	Sperm count (millions)	Abnormality (%)
1	49.4	0.27	44.80	29.8
2	40.6	0.24	45.80	22.0
3	46.8	0.35	60.75	16.9
4	46.0	0.33	46.75	19.3
Average	45.7	0.30	49.52	22.0

It is interesting to indicate that during the study, captures were almost exclusively of *P. occidentalis*. This seems to indicate that during reproduction activity, penaeid species disperse from each other, as it has been reported for *P. setiferus* and *P. aztecus* (Chamberlain and Lawrence, 1983).

In the southeast coast of Chira island (Lagartero and Lagartito beaches), two *P. occidentalis* females in stage 4 of maturation with spermatophore residues, and one *P. stylirostris* female in stage 4, but not impregnated, were found. This particular finding could be an indication that activities like the one monitored near Curazao beach, are occurring in other areas of Gulf of Nicoya, where *P. occidentalis* and *P. stylirostris*

reproduce. Only through constant observation it will be defined where and when these reproductive events occur. Knowing the location of spawning grounds has a great importance for the establishment of permanent protection areas, that contribute to the balance between exploitation and conservation.

Mating of *P. occidentalis* in the study area occurred during early afternoon hours. Impregnated females were found in samples taken at 12:00. This finding indicates that the reproductive activity was very intense because open thelycum shrimp generally mate at dusk (Chamberlain and Lawrence, 1983). Open thelycum shrimps mate a few hours before spawning, and the presence of a sperm mass, at the moment of spawning, is a prerequisite for fertilization. On the contrary, closed thelycum females like *P. aztecus* (Chamberlain and Lawrence, 1983), *P. notialis*, *P. subtilis*, *P. brasiliensis* (Martínez et al., 1984), mate before ovary development and store sperm in their seminal receptacle (Browdy, 1992). Sperm mass removal by females, in the present study, is probably a consequence of stress associated with capture and transport. However, other wild open thelycum penaeids like *P. setiferus* (Bray et al., 1983) and *P. schmitti* (Martínez et al., 1984) do not express such behavior, releasing fertile eggs after capture and transport.

The spawning behavior is similar to that observed in *P. vannamei* grown in ponds (unpublished data) and *S. ingentis* (Pillai et al., 1988). However, the description by phases presented in this document contributes to a better definition of the process.

Female removal at the moment of spawning for *in vitro* assays resulted in the partial release of eggs. On this matter, wild *P. occidentalis* demonstrated to be highly sensitive to the technique; on the contrary, wild *S. ingentis* (Pillai et al., 1988), *P. vannamei* and *P. stylirostris* matured in the laboratory (unpublished data), generated a massive release of eggs by this technique.

Cortical reaction events are similar to those described for the closed thelycum species, *S. ingentis* (Pillai and Clark, 1987) and *P. aztecus* (Clark et al., 1980). To our knowledge, for open thelycum shrimps, the cortical events have been described only for *P. setiferus* (Clark et al., 1980). Our findings on *P. occidentalis* indicate that at contact with seawater eggs experience a massive release of cortical rods, lifting the vitelline envelope. Sperm cells are observed in primary binding to the vitelline envelope; with the release of rods, eggs reduce their size from 280 µm to 220 µm. Finally, rods dissipate and the hatching envelope develops. Sperm are not required for the activation of these events.

During sperm-egg interaction (Clark et al., 1981) or *in vitro* activation with egg water (Griffin et al., 1988), *S. ingentis* sperm experience an acrosomal reaction. In *P. aztecus* the reaction is different and it has been observed during the sperm-egg interaction (Clark et al., 1980). In *P. stylirostris*, the acrosomal reaction seems to be

characterized by spike depolymerization, followed by acrosomal vesicle exocytosis (Clark, W. and Griffin, F., personal communication).

In this research, during the sperm-egg interactions of *P. occidentalis*, no sperm were observed developing an acrosomal reaction. Only the primary binding between sperm and the vitelline envelope of eggs occurred massively. In *S. ingentis* most sperm experience an acrosome reaction when cells are mixed *in vitro*. To our knowledge, there are no references about acrosomal reaction in open thelycum penaeids; therefore, the analysis of our observations is limited. Based on studies with *S. ingentis*, it would be expected that a massive acrosome reaction during the interaction between wild eggs (good quality) and wild sperm of *P. occidentalis* would occur, but it did not happen that way. It seems that fertilization in open thelycum shrimps has particularities which have not yet been understood.

Wild males of *P. occidentalis* showed spermatophore quality parameters similar to those obtained for wild *P. setiferus* (sperm count = 45 million, abnormality = 23%; Alfaro, 1990). *P. occidentalis* spermatophore weight (0.30 g) is considerably heavier than the average value registered for *P. setiferus* (0.14 g). The size of these units may be the cause for the unsuccessful manual ejaculation, which caused damage to the reproductive system in every tested male (n = 24). This problem does not occur with *P. vannamei*, *P. stylirostris*, and *P. setiferus*, which can be successively ejaculated. This particularity of *P. occidentalis* is a limitation for male reutilization in future stock enhancement programs using cultured post-larvae.

Acknowledgements

This research is part of the project: White Shrimp, supported by CONICIT – Ley de Pesca – Escuela de Ciencias Biológicas, UNA, and the project: Controlled Reproduction of Shrimp, supported by the programs UNA-JICA and UNA-LUW. We thank Vicerrectoría de Investigación, UNA, Chira island community, Criadero de Camarones de Chomes S.A., and to the students, Alex Molina, Sidey Arias and Gerardo Zúñiga for their cooperation.

References

Alfaro, J., 1990. A contribution to the understanding and control of the male reproductive system melanization disease of brood stock *Penaeus setiferus*. Master Thesis, Texas A and M University, College Station, Texas, USA.

Alfaro, J., 1992. Reproductive quality evaluation of male *Penaeus stylirostris* from a grow-out pond. J. World Aquacult. Soc. 24 (1), 6 – 11.

Bray, W. A., Chamberlain, G. W., Lawrence, A. L., 1983. Observations on natural and artificial insemination of *Penaeus setiferus*. Proc. 1st Int. Conf. on Warm Aquaculture, Crustacea. Hawaii, pp. 392 – 405.

Boschi, E., 1974. Biología de los crustáceos cultivables de América Latina. Simp. FAO/CARPA Sobre Acuacul. en Amer. Lat., Uruguay, 23 pp.

Browdy, C., 1992. A review of the reproductive biology of *Penaeus* species: Perspectives on controlled shrimp maturation systems for high quality nauplii production. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 22 – 51.

Carranza, F. P., 1985. Distribución y abundancia del recurso de camarón blanco y alternativas de aprovechamiento mediante un método de pesca artesanal en el Golfo de Nicoya. Tesis de maestría, Universidad Nacional Autónoma de México, D. F., México, 60 pp.

Chamberlain, G. W., 1988. Stepwise investigation of environmental and nutritional requirements for reproduction of penaeid shrimp. PhD Thesis, Department of Wildlife and Fisheries Sciences, Texas A & M University, USA.

Chamberlain, G. W., Lawrence, A. L., 1983. Reproductive activity and biochemical composition of *Penaeus setiferus* and *Penaeus aztecus* in the Gulf of Mexico. TAMU-SG-84-203, College Station, Texas, 35 pp.

Clark, W. H., Kleve, M. G., Yudin, A. I., 1981. An acrosome reaction in natantian sperm. J. Exp. Zool. 218, 279 - 291.

Clark, W. H., Lynn, J. W., Yudin, A. I., Persyn, H., 1980. Morphology of the cortical reaction in the eggs of *Penaeus aztecus*. Biol. Bull. 158, 175 - 186.

Clark, W. H., Yudin, A. I., Griffin, F. J., Shigekawa, K., 1984. The control of gamete activation and fertilization in the marine penaeidae, *Sicyonia ingentis*. In: Engels, W. (Ed.), Advances in invertebrate reproduction 3. Elsevier Science Publishers. New York, pp. 459 – 472.

DeVries, M. C., Epifanio, C. E., Dittel, A. I., 1983. Reproductive periodicity of the tropical crab *Callinectes arcuatus* Ordway in Central America. Est. Coast. Shelf Sci. 17, 709 - 716.

Epifanio, C. E., Dittel, A. I., 1984. Seasonal abundance of brachyuran crab larvae in a tropical estuary: Gulf of Nicoya, Costa Rica, Central America. Estuaries 7 (4B), 501 - 505.

García, S., LeReste, L., 1981. Life cycles, dynamics, exploitation and management of coastal penaeid shrimp stocks. FAO Fisheries Technical Report No. 203, 116 pp.

Griffin, F. J., Shigekawa, K., Clark, W. H., 1988. Formation and structure of the acrosomal filament in the sperm of *Sicyonia ingentis*. J. Exp. Zool. 246, 94 - 102.

Hudinaga, M., 1942. Reproduction, development and rearing of *Penaeus japonicus* Bate. Jpn. J. Zool. 10, 305 - 393.

Imai, T. (Ed.), 1980. Aquaculture in shallow seas: progress in shallow sea culture. A. A. Balkema, Rotterdam, Holanda.

Leung-Trujillo, J., Lawrence, A. L., 1985. The effect of eyestalk ablation on spermatophore and sperm quality in *Penaeus vannamei*. J. World Maric. Soc. 16, 258 - 266.

Leung-Trujillo, J., Lawrence, A. L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65, 363 - 370.

Lynn, J. W., Clark, W. H., 1987. Physiological and biochemical investigations of the egg jelly release in *Penaeus aztecus*. Biol. Bull. 173, 451 - 460.

Martínez, L. S., Torres, M., Remolina, R., Maldonado, J., 1984. Reproducción artificial de cinco especies diferentes de camarón marino para la obtención de semilla como alternativa en el repoblamiento de áreas naturales y desarrollo acuícola. Centro de Investigaciones Pesqueras, INDERENA, Colombia, 83 pp.

Motoh, H., 1981. Studies on the fisheries biology of the giant tiger prawn, *Penaeus monodon* in the Philippines. Technical paper No. 7, SEAFDEC, Filipinas.

Palacios, J. A., Rodríguez, J. A., Angulo, R. A., 1992. Estructura poblacional de *Penaeus stylirostris* (Decapoda:Penaeidae), en el Golfo de Nicoya, Costa Rica. Rev. Biol. Trop. (in press).

Peterson, C. L., 1960. La oceanografía física del Golfo de Nicoya, Costa Rica, un estuario tropical. Inter. Amer. Trop. Tun. Comm. Bull. 4 (4), 139 - 216.

Pillai, M. C. , Clark, W. H., 1987. Oocyte activation in the marine shrimp, *Sicyonia ingentis*. J. Exp. Zool. 244, 325 - 329.

Pillai, M. C., Clark, W. H., 1988. Hatching envelope formation in shrimp (*Sicyonia ingentis*) ova: origin and sequential exocytosis of cortical vesicles. Tissue Cell 20 (6), 941 - 952.

Pillai, M. C., Griffin, F. J., Clark, W. H., 1988. Induced spawning of the decapod crustacean *Sicyonia ingentis*. Biol. Bull. 174, 181 - 185.

Talbot, P., Howard, D., Leung-Trujillo, J., Lee, T. W., Li, W-Y. , Ro, H., Lawrence, A. L., 1989. Characterization of male reproductive tract degenerative syndrome in captive penaeid shrimp (*Penaeus setiferus*). Aquaculture 78, 365 - 377.

Valdés, J., Brenes, C. L., Solís, E., Mendelewicz, M., 1987. Propiedades físico químicas de las aguas del Golfo de Nicoya, Costa Rica. Ing. Cienc. Quim. 11 (1), 21 - 25.

Vitola, M. M., 1985. Camarones peneidos (Decapoda:Natantia) del Golfo de Nicoya, Costa Rica: Un análisis de su distribución y densidad. Tesis de Maestría, Universidad de Costa Rica, San José.

Chapter 3

Interaction of Bacteria and Male Reproductive System Blackening Disease of Captive *Penaeus setiferus*

J. Alfaro[1], A. L. Lawrence[2] and D. Lewis[3]

[1] Escuela de Ciencias Biológicas, Universidad Nacional, Heredia, Costa Rica.
[2] Department of Wildlife and Fisheries Sciences, Texas Agricultural Experimental Station, Texas A&M University, P.O. Drawer Q, Port Aransas, TX 78373.
[3] Department of Veterinary Microbiology and Parasitology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843.

Published in: Aquaculture (1993), 117: 1 – 8

Abstract

The genital apparatus of male penaeid shrimp, *Penaeus setiferus*, blackens with resulting detrimental effects on mating for production of larvae when animals are kept in controlled maturation/reproduction situations. A progressive, melanized condition of the male reproductive tract was shown to be associated with bacterial infection. At least three different species (*Vibrio alginolyticus*, *Pseudomonas putrefaciens*, and an unclassified strain), were isolated from damaged tissues and successfully developed the same signs in challenge experiments. It is suggested that the condition could be a progressive syndrome with bacterial invasion perhaps only in the advanced stages, or that more than one etiology may be involved in deterioration and blackening of *P. setiferus* reproductive system.

Introduction

Pathological conditions associated with the male reproductive system of penaeid shrimp have been reported. These conditions frequently affect Gulf of Mexico white shrimp, *Penaeus setiferus*, when this species is held in captivity. Reproductive tissue darkening has been also documented from *P. vannamei*, *P. stylirostris*, and the fresh water prawn, *Macrobrachium rosenbergii* (Chamberlain et al., 1983; Harris and Sandifer, 1986). It appears that *P. setiferus* is particularly susceptible to these conditions, which are detrimental to larvae production since they render males infertile.

Signs of conditions affecting male reproductive system were described previously by Chamberlain et al. (1983), Leung-Trujillo and Lawrence (1987), Chamberlain (1988), and Talbot et al. (1989). Until now the cause of these conditions has not been established. The study upon which this report was based, was designed to evaluate the possible interaction between bacterial infection and a male reproductive system blackening condition of *Penaeus setiferus*.

Materials and methods

Broodstock and experimental conditions

Sexually mature *Penaeus setiferus* males with a mean weight of 40 ± 5.2 g (mean ± SEM) were obtained by trawling off Port Aransas (27°5' N, 97°3, W), Texas. Animals were transported in chilled (22 °C) and oxygenated seawater to the Texas A&M University Laboratory at Port Aransas, where they were gradually acclimated to the culture water (temperature = 30 °C, salinity = 31 ppt).

Shrimp were stocked at 3.3 animals m^{-2} in 2.4 m diameter light blue fiberglass tanks. Water flow was continuous and adjusted to 15 l min^{-1} using a semi-closed water recirculation system without sterilization. Broodstock were fed at 3% body weight day^{-1} of a pelleted prepared broodstock diet (60% protein; 10% lipid).

Hydrological parameters were optimum and stable during the experimental period. Water temperature was 29 ± 1 °C, salinity 30 ± 1 ppt, dissolved oxygen 6.4 ± 0.3 mg l^{-1}, and ammonia-nitrogen 0.107 ± 0.053 mg l^{-1}.

Bacteria isolation and characterization

Three different types of reproductive tissue were classified based on tissue appearance: A) tissue from wild males within 12 days of capture, showing spermatophores, ampoules, and vas deferens with normal coloration, B) tissue from wild males more than 32 days post capture, showing deteriorated spermatophores, but no black pigment on ampoules or vas deferens, C) tissue from wild males more than 12 days post capture, showing black pigment on spermatophores, ampoules, and vas deferens. Various regions of the reproductive system were processed to recover bacteria as follows. The male genital apparatus was aseptically dissected in sterile petri dishes, then the system was divided into ampoule, distal, medial vas deferens segments 2A and 2B (Talbot et al., 1989). Some spermatophores were removed from their ampoules. Reproductive system sections were then macerated in 2 ml sterile saline (0.85%) and homogenized in a sterile blender.

Homogenized materials were streaked on trypticase soy agar (Difco Laboratories, Detroit, MI 48232) containing 2% sodium chloride (TSA) and incubated at 25 °C for 2 days. Bacterial growth was observed 24 h after inoculation; negative plates were incubated for an additional five days to detect slow-growing bacteria.

Bacterial characterization was performed using the API 20E System (Analytab Products, 200 Express Street, Plainview, NY 11803) as recommended by Lightner (1983).

Infectivity experiments

Bacteria recovered from black reproductive sections were cultured in trypticase soy broth (TSB, Difco Laboratories) containing 2% sodium chloride at 25 °C for 24 h. Three challenge systems were evaluated (n= 6 males per system): intra muscular injection (26-gauge tuberculin syringes) of 0.1 ml 5-fold-diluted saline (0.85%)-resuspended isolate 1; surface exposure (prepared by centrifugation of broth) of isolate 1 applied with sterile cotton swab to gonopores; and injection into the gonopore of 0.05 ml 5-fold-diluted saline-resuspended isolate 1. Controls were provided using the same challenge systems using sterile TSB-salines (n= 6 males per system). A fourth challenge system was then evaluated: gonopore injection of 0.05 ml 50-fold-diluted saline-resuspended isolate 1 (n= 10), isolate 2 (n= 5), and isolate 4 (n= 5); sterile TSB-salines were used as controls (n= 5 males per isolate). Broth was eliminated by centrifugation prior to bacterial resuspension and dilution. Males selected for these experiments presented deteriorated reproductive systems (more than 54 days post capture) with no visible tan to black coloration.

Results

Isolation of bacteria from male reproductive systems

Bacterial isolation attempts revealed that only black reproductive tissue sections (type C tissue) contained bacteria (Table 1). Inoculated plates from black reproductive sections gave positive and massive growth of pure colonies at 25 °C in 24 h from incubation. Neither type A tissue or type B tissue produced bacterial growth.

Table 1. Bacteria recovered from different sections of reproductive systems of *Penaeus setiferus*.

Reproductive tissue condition[a]	Section	n	Bacteria growth[b]
Type A	SP	3	(-)
	AM/SP	1	(-)
Type B	SP	6	(-)
	AM/SP	2	(-)
	AM	1	(-)
	MV2A	1	(-)
	MV2B	1	(-)
Type C	SP	2	(+)
	AM/SP	1	(+)
	DV	1	(+)
	MV2A	1	(+)
	MV2B	1	(+)

[a] Type A= tissue showing normal coloration. Type B= tissue showing deteriorated spermatophores, but no black pigment on ampoules or vas deferens. Type C= tissue showing black pigment.

[b] Recovering was performed in TSA 2% NaCl incubated at 25 °C for 6 days. AM= ampoule, DV= distal vas deferens, MV2A= medial vas deferens 2A, MV2B= medial vas deferens 2B, SP= spermatophore.

Organisms were identified based on morphological and biochemical characteristics of isolates (Table 2) and the keys of 20E Analytical Profile Index (Analytab Products), Bullock (1971), Lightner (1983), and Frerichs and Hendrie (1985). Identification suggests that isolate 1 and 3 are *Vibrio alginolyticus*, isolate 4 is *Pseudomonas putrefaciens*, and isolate 2 could not be identified with the available information.

Table 2. Characteristics of bacteria isolated from black reproductive systems of *Penaeus setiferus*.

Reaction[a]	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Morphology	rod	Rod	rod	rod
Gram stain[b]	-	-	-	-
MacConkey growth	+	-	+	+
Cytochrome oxidase	+	+	+	+
Motility	+	+	+	+
Glucose fermentation	+	+	+	-
Glucose oxidation	+	+	+	-
O-nitrophenyl-ß-d-galactoside	-	+	-	-
Arginine hydrolysis	-	-	-	-
Lysine hydrolysis	+	+	+	-
Ornithine hydrolysis	-	-	-	+
Citrate hydrolysis	+	-	+	+
H_2S production	-	-	-	+
Urea hydrolysis	-	-	-	+
Tryptophane hydrolysis	-	-	+	-
Indol	+	+	+	-
Voges-Proskauer	-	-	-	-
Gelatin hydrolysis	+	-	+	+
Mannitol fermentation	+	+	+	-
Inositol fermentation	-	-	-	-
Sorbitol fermentation	-	+	-	-
Rhamnose fermentation	-	-	-	-
Sucrose fermentation	+	+	+	-
Melibiose fermentation	-	-	-	-
Amygdalin fermentation	+	+	+	-
Arabinose fementation	-	-	-	-
Nitrate reduction	-	-	-	+
Nitrogen gas	-	-	-	-
Salt requirement	+	-	+	-
Growth at 35 °C	+	-	+	+

[a] Using Analytab Products, Inc. system.

[b] Using BBL Gram Stain Kit, and Fisher Gram Slides for control. *Staphylococcus aureus*: Gram-positive control; *Escherichia coli*: Gram-negative control.

Bacteria challenging

Attempts to produce blackening of reproductive systems were initially accomplished by challenging with *Vibrio alginolyticus* (isolate 1). Four different infectivity mechanisms were evaluated.

Injection beneath third abdominal segment and surface exposure (by swab) did not blacken reproductive tissue. Gonopore injection of 5-fold diluted culture caused 83% mortality in less than 24 h with no blackening of reproductive tissue. Controls did not induce the condition.

Challenging by injecting 50-fold-diluted 24-h culture into terminal ampoules did initiate swelling and blackening of reproductive systems. Induction of male reproductive blackening was achieved not only with *Vibrio alginolyticus*, but also with *Pseudomonas putrefaciens* (isolate 4), and isolate 2 (Table 3). Induced blackening begins with swelling of ampoules, then spermatophores become black, and in less than 6 days from injection blackening has extended to medial vas deferens. Injection of 0.05 ml sterile TSB-saline into terminal ampoules (controls) did not initiate the condition.

Table 3. Infectivity response to initiate blackening of reproductive systems of *Penaeus setiferus*.

Condition	No. challenged Males	No. shrimp developing blackening[c]
V. alginolyticus[a]	10	10
Control[b]	5	0
Isolate 2[a]	5	4
Control[b]	5	0
P. putrefaciens[a]	5	3
Control[b]	5	0

[a] 0.05 ml 50-fold diluted 24-h TSB culture injected through left gonopore.

[b] 0.05 ml TSB-saline control injected through right gonopore.

[c] Swelling and blackening developed during the following 6 days after injection.

Discussion

Under our experimental conditions, *P. setiferus* rapidly became infertile as described in other studies (Bray et al., 1985; Leung-Trujillo and Lawrence, 1987; Chamberlain, 1988; Talbot et al., 1989).

Isolation of bacteria from type C tissue (black spermatophores, ampoules, and vas deferens) suggests association of bacterial infections with blackened reproductive systems. Types A and B tissue were aseptic, indicating that normal reproductive tissue and reproductive systems in deterioration, as described by Talbot et al. (1989), do not represent bacterial infection. The visual differences in reproductive system quality between the three types of tissues could indicate a progressive syndrome, with bacterial infection becoming involved in advanced stages, or that more than one etiology may be involved in deterioration and blackening of *P. setiferus* reproductive system.

The infectivity experiments indicate that black reproductive system signs can be induced by challenging with *Vibrio alginolyticus*, *Pseudomonas putrefaciens*, and isolate 2. Each species induced the condition at different levels of positive cases (Table 3); *V. alginolyticus* was the best isolated inducer (100% of cases). *Vibrio* spp. are a normal part of the microflora of pond and raceway-reared shrimp (Vanderzant et al., 1971; Lightner, 1985) and are opportunistic pathogens (Lightner, 1977). Both organisms (*Vibrio* and *Pseudomonas*) have been frequently isolated from other cultured prawn and penaeid shrimp diseases (Lewis, 1973; Delves-Broughton and Poupard, 1976; Lightner and Lewis, 1975; Lightner, 1983). Shell disease of crabs, lobsters, and penaeid shrimp, has been associated with those chitinoclastic genera (Cook and Lofton, 1973; Malloy, 1978; Cipriani et al., 1980).

The evidence presented here and our current understanding indicate that bacterial infection interacts with blackening of spermatophores, ampoules, and vas deferens. Some opportunistic bacteria, of at least three different genera, penetrate the ampoule through gonopores. The invasion is accelerated by artificial ejaculation (Chamberlain et al., 1983; Sandifer et al., 1984), but the condition also appears in non-ejaculated animals suggesting that invasion may occur in shrimp with impaired defense mechanisms resulting from stressful culture conditions.

Micro-organisms reach the surface of spermatophore acellular matrix and vas deferens, where black depositions appear (Harris and Sandifer, 1986). They find a good supporting chitinous substrate for their propagation (Benton, 1935), and shrimp's defense system is activated. Melanin, which is a defense and toxic molecule (Graham et al., 1978; Bang, 1983), is produced by the shrimp's prophenoloxidase system. This mechanism plays a major role in foreign substance recognition by the host (Ashida and Soderhall, 1984; Soderhall and Smith, 1986), and it has been recently confirmed in *P. japonicus*, *P. monodon*, *Macrobrachium rosenbergii*, and *Palaemon adspersus* (Tsing et al., 1989).

Dougherty and Dougherty (1989) found black pigment droplets, identified as melanin, not only confined to the surface tissue of spermatophores, but also in the sperm mass matrix of *P. vannamei*. These authors did not find bacteria using light and electron

microscopy. It is of course possible that similar gross pathology could have different etiology in penaeid shrimp.

Acknowledgements

We thank the Texas A&M University Mariculture Program staff and graduate students: Bill Bray, Joanna Leung-Trujillo, Frank Castille, Tzachi Samocha, Karen Hall, He Haiqi, Marco Araujo, Jose Valverde, Luis Mena, Allen Davis, Tammy Thompsen, and James Biedenbach. To Matt Mireles who helped us in some experiments and animal maintenance; to Vicki Chandler, project secretary, for her continuous collaboration, and Mr. Jim Tucker, Captain of "Tuff Enouff".

References

Ashida, M., Soderhall, K., 1984. The prophenoloxidase activating system in crayfish. Com. Biochem. Physiol. 77B (1), 21 - 26.

Bang, F. B., 1983. Crustacean disease responses. In: Provenzano, A. (Ed.), The biology of crustacea. v.6. Pathobiology. Academic Press, Inc., New York, NY, USA, pp. 113 - 149.

Benton, A. G., 1935. Chitinovorous bacteria -a preliminary survey. J. Bacteriol. 29, 449 - 465.

Bray, W. A., Leung-Trujillo, J. R., Lawrence, A. L., 1985. Preliminary investigation on the effects of temperature, bacterial inoculation, and EDTA on sperm quality in captive *Penaeus setiferus*. J. World Maric. Soc. 16, 250 - 257.

Bullock, G. L., 1971. Diseases of fishes. Book 2B. In: Sniesko, S., Axelrod, H. (Eds.), Identification of fish pathogenic bacteria. T.F.H. Publications, New York, NY, USA, 43 pp.

Chamberlain, G. W., 1988. Stepwise investigation of environmental and nutritional requirements for reproduction of penaeid shrimp. Ph.D. Thesis, Department of Wildlife and Fisheries Sciences, Texas A & M University, USA.

Chamberlain, G. W., Johnson, J. K., Lewis, D. H., 1983. Swelling and melanization of the male reproductive system of captive adult penaeid shrimp. J. World Maric. Soc. 14, 135 - 136.

Cipriani, G. R., Wheeler, R. S., Sizemore, R. K., 1980. Characterization of brown spot disease of Gulf Coast shrimp. J. Invertebr. Pathol. 36, 255 - 263.

Cook, D. W., Lofton, S. R., 1973. Chitinoclastic bacteria associated with shell disease in *Penaeus* shrimp and the blue crab. J. Wildl. Dis. 9, 154 - 159.

Delves-Broughton, J., Poupard, C. W., 1976. Disease problems of prawns in recirculation systems in the U.K. Aquaculture 7, 201 - 217.

Dougherty, W. J., Dougherty, M. M., 1989. Electron microscopical and histochemical observations on melanized sperm and spermatophores of pond-cultured shrimp, *Penaeus vannamei*. J. Invertebr. Pathol. 54, 331 - 343.

Frerichs, G. N., Hendrie, M. S., 1985. Bacteria associated with diseases of fish. In: Collins, C. H., Grange, J. M. (Eds.), Isolation and identification of micro-organisms of medical and veterinary importance. Academic Press, Orlando, FL, USA, 387 pp.

Graham, D. G., Tiffany, S. M., Vogel, F. S., 1978. The toxicity of melanin precursors. J. Invest. Dermatol. 70, 113.

Harris, S., Sandifer, P., 1986. Sperm production and the effects of electrically induced spermatophore expulsion in the prawn *Macrobrachium rosenbergii* (De Man). J. Crust. Biol. 6 (4), 633 - 647.

Leung-Trujillo, J. R., Lawrence, A. L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65, 363 - 370.

Lewis, D. H., 1973. Response of brown shrimp to infection with *Vibrio* sp. Proc. 4th Ann. Workshop, World Maric. Soc., pp. 333 - 338.

Lightner, D. V., 1977. Crustacean diseases. In: Sinderman, C. J. (Ed.), Disease Diagnosis and Control in North American Marine Aquaculture. Elsevier Scientific Publishing Co., New York, NY, USA, pp. 8 - 77.

Lightner, D. V., 1983. Diseases of cultured penaeid shrimp. In: McVey, J. P. (Ed.), CRC handbook of mariculture, vol. 1, Crustacean aquaculture. CRC Press, FL, USA, pp. 289 - 320.

Lightner, D. V., 1985. A review of the diseases of cultured penaeid shrimps and prawns with emphasis on recent discoveries and developments. In: Taki, Y., Primavera, H. J., Llobrera, J. A. (Eds.), Proc. 1st Int. Conf. on the Culture of Penaeid Prawns/Shrimp, Aquaculture Department, SEAFDEC, Iloilo, Philippines, pp. 79 - 103.

Lightner, D. V., Lewis, D. H., 1975. A septicemic bacterial disease syndrome of penaeid shrimp. Mar. Fish. Rev. 37, 25 - 28.

Malloy, S., 1978. Bacteria induced shell disease of lobsters (*Homarus americanus*). J. Wildl. Dis. 14, 2 - 9.

Sandifer, P. A., Lawrence, A. L., Harris, S. G., Chamberlain, G. W., Stokes, A. D., Bray, W. A., 1984. Electrical stimulation of spermatophore expulsion in marine shrimp, *Penaeus* spp. Aquaculture 41, 181 - 187.

Soderhall, K., Smith, V. J., 1986. The prophenoloxidase activating system: the biochemistry of its activation and role in arthropod cellular immunity, with special reference to crustaceans. In: Brehelin, M. (Ed.), Immunity in invertebrates. Springer-Verlag, New York, NY, USA, pp. 208 - 223.

Talbot, P., Howard, D., Leung-Trujillo, J., Lee, T. W., Li, W-Y., Ro, H., Lawrence, A. L., 1989. Characterization of male reproductive tract degenerative syndrome in captive penaeid shrimp (*Penaeus setiferus*). Aquaculture 78, 365 - 377.

Tsing, A., Arcier, J., Brehelin, M., 1989. Hemocytes of penaeid and palaemonid shrimps: morphology, cytochemistry, and hemograms. J. Invertebr. Pathol. 53, 64 - 77.

Vanderzant, C., Nickelson, R., Judkins, P. W., 1971. Microbial flora of pond-reared brown shrimp (*Penaeus aztecus*). Appl. Microbiol. 21(5), 916 - 921.

Chapter 4

Reproductive Quality Evaluation of Male *Penaeus stylirostris* from a Grow-Out Pond

J. Alfaro

Escuela de Ciencias Biológicas, Universidad Nacional, Heredia, Costa Rica

Published in: J. World Aquacult. Soc. (1993), 24 (1): 6 – 11

Abstract

Several male *Penaeus stylirostris* were selected from a 3-ha commercial earthen pond and were individually evaluated for reproductive performance. Indicators measured were compound spermatophore weight, sperm count, and sperm abnormalities. It was found that spermatophore quality was significantly better for 30-40 g shrimp than for 20-30 g shrimp ($P<0.05$). The higher frequency of abnormalities measured in younger males and the inverse relationship between abnormalities and sperm count indicate that the vas deferens could be the tissue responsible for producing highly abnormal immature semen. We propose that male maturation has at least three independently controlled levels of organization: testes maturation, vas deferens maturation, and spermatophore synthesis. The individual evaluation showed that each male followed a particular response in reproductive quality. Changes in spermatophore weight were not an indicator of sperm density within spermatophores. Male reproductive tract degenerative syndrome (MRTDS) and male reproductive system melanization (MRSM) did not develop in any shrimp during our experiments.

Introduction

The progress of shrimp mariculture depends on a better understanding of specific biological aspects, particularly in reproductive biology, to ensure that adequate numbers of laboratory-reared postlarvae can be produced at high levels of efficiency. Although significant improvements in controlled reproduction have been made in the past 5 to 10 years, much of the interest has focused on female maturation (Primavera, 1985; Chamberlain, 1988). Knowledge about male maturation and semen quality is fragmentary (Leung-Trujillo and Lawrence, 1987a).

The evaluation of reproductive quality in male penaeid shrimp was first reported by Leung-Trujillo and Lawrence (1985), who measured spermatophore weight, sperm count, and percentages of live and abnormal sperm for evaluating the effect of eyestalk ablation in *P. vannamei*. Using the same parameters, Leung-Trujillo and Lawrence (1987a) studied a decline in sperm quality in *P. setiferus* held in captivity and Bray et al. (1985) used the same approach to evaluate the effect of water temperature, EDTA, and *Vibrio* bacterin on sperm quality in captive *P. setiferus*. Chamberlain (1988) assessed the effect of dietary rancidity and vitamin E on gonadal maturation of *P. setiferus*. Leung-Trujillo and Lawrence (1987b) evaluated spermatophore redevelopment times in wild *P. stylirostris*, *P. vannamei*, and *P. setiferus*; and Leung-Trujillo and Lawrence (1988) studied the effect of ascorbic acid on reproductive quality in *P. vannamei* using the previous mentioned quality indicators. Recently, Alfaro (1990) measured spermatophore weight, sperm count, and percentage of abnormalities for evaluating nutritional variations

on the onset of the male reproductive tract degenerative syndrome (MRTDS) of *P. setiferus* (Talbot et al., 1989).

Thus, the knowledge that has accumulated on this subject is based on the analysis of various treatment protocols, with little information available concerning the way a male produces spermatophores. The purpose of this study is to evaluate reproductive quality during successive spermatophore regenerations for individual male *P. stylirostris* selected from commercial grow-out ponds.

Materials and methods

Animals

Male *P. stylirostris* with spermatophores (n = 12) were selected from a commercial 3-ha earthen pond during the density adjusting phase at 2.5 months post-stocking. Shrimp were reared at a stocking density of 5 shrimp m^{-2} with a minimum water exchange of 10% daily using inorganic fertilization and supplemental feeding in a polyculture strategy, but primarily using *P. vannamei*. Selected males were packed in plastic bags of oxygenated and chilled seawater (25 °C). Bags were transported in ice chests to the Aquaculture Laboratory at Universidad Nacional, Heredia, where the shrimp were acclimated to the culture water.

Experimental Conditions

Males were stocked (12 shrimp m^{-2}) in a 1.0 m^2 recirculating culture unit with external biological filter and automatic control of temperature (Earth Shyokai Co., Ltd, Tokyo, Japan). Water exchange was limited to replacement evaporative loss. A fresh-frozen diet initially consisting of shrimp heads (*P. occidentalis*), marine bloodworms (*Nereis* sp.), fish (*Opistonema* sp.), and clams was provided at a ratio of 1:1:1:1, respectively, at 10% of wet body weight per day. After the third spermatophore regeneration, the diet was completely replaced by fresh-frozen squid. The total daily ration was distributed over 4 feedings at 09:00, 12:00, 14:00, and 17:00 h. Salinity was maintained at 32 ppt, temperature at 29 °C, pH at 7.7, and total NH_4-N level at less than 0.01 mg l^{-1}.

Experimental Design

Selected males were initially analyzed immediately after stocking in the recirculating unit. Spermatophores were removed by manual ejaculation following a modified procedure of that described by King (1948) in which the spermatophore was partially ejected with gentle pressure and then completely expelled employing disinfected fine tweezers.

Total body weight was measured to the nearest 0.1 g and compound spermatophore weight to the nearest 0.01 g. In addition, sperm count per compound spermatophore and percentage of abnormal sperm were determined. Sperm count was performed by homogenizing the compound spermatophore in a glass tissue grinder with 3.0 ml of artificial seawater (Cavanaugh, 1956). The suspension was mixed several times to insure homogeneity, and 3 samples were counted using the total 25 fields of a hemacytometer (0.0001 ml).

The percentage of abnormal sperm was obtained by recording the number of normal sperm, i.e. with a spherical body and an elongate spike (Talbot et al., 1989), and number of abnormal sperm, i.e. with malformed heads and bent or missing spikes for at least 100 sperm cells. Percent abnormal sperm were calculated as follows:

% abnormal sperm

= 100 [abnormal cells/ (abnormal cells + normal cells)]

At stocking, five males were individually marked by cutting the uropods in a similar way to that described by Makinouchi and Primavera (1987). Females were not stocked in order to allow the evaluation of male reproductive performance without the enhancing effects introduced by female presence. Marked males were manually ejaculated every 13 days, until 5 regenerations were obtained.

Initial data on shrimp size were statistically analyzed for significant differences among means using the t-test for independent samples with unequal variance (Ott, 1984). Sperm count data were transformed using squared root of Y + 3/8 (Bray et al., 1985) and an arcsine transformation was used in the analysis of abnormal sperm percentages (Leung-Trujillo and Lawrence, 1985) to make the variance independent of the mean. Data on sperm counts and abnormalities were analyzed for linear regression and correlation (Ott, 1984).

Results

Penaeus stylirostris grown in an earthen pond were found to produce spermatophores in individuals larger than 23.6 g (total length = 100 mm). Spermatophores were significantly smaller in weight ($P<0.05$) for 20-30 g (100-111 mm) shrimp than for 30-40 g (112-116 mm) shrimp. In addition, spermatophore quality was significantly improved ($P<0.05$) in the 30-40 g shrimp compared to the 20-30 g shrimp (Table 1). A linear regression between sperm abnormalities and sperm count was calculated, including the initial data and the laboratory regeneration data. It indicates an inverse relationship, which is represented by the following equation:

$$Y = 63.4 - 3.4 X,$$

where Y is the percent abnormal sperm and X is the sperm count in millions. A correlation coefficient of -0.52 was calculated. Confidence intervals were 63.4 ± 12.3 (intercept) and

−3.4 ± 2.5 (slope; $P < 0.05$). The linear relationship between these two parameters was statistically significant ($P < 0.05$) in a test for slopes.

Table 1. Comparison of compound spermatophore weight, sperm count, and abnormalities between size classes for *Penaeus stylirostris* selected from a commercial grow-out pond. Data are represented as mean ± standard error (N = 4). Means with different letters are statistically different ($P < 0.05$).

Parameter	Weight class	
	20-30 g	30-40 g
Spermatophore weight (g)	$0.03^a \pm 0.01$	$0.06^b \pm 0.03$
Sperm count (million)	$1.62^a \pm 1.02$	$4.83^b \pm 2.10$
Abnormal sperm (%)	$75^a \pm 12$	$50^b \pm 12$

The individual evaluation of spermatophore quality is presented for 5 regenerations. Table 2 shows data on sperm count; Table 3 summarizes the spermatophore weight data, and the percentages of abnormal sperm are shown in Table 4. Individual shrimp evaluation indicates that each male generated spermatophores within 13 days after each manual ejaculation. The only exception to this statement was male D at its fifth regeneration time (Table 2).

Table 2. Sperm counts (million) for five spermatophore regenerations of individually marked *Penaeus stylirostris*.

Male:	Regeneration number				
Body weight (g)	1	2	3	4	5
A: 37	3.18	2.76	4.89^a	4.53	4.26
B: 35	6.84^a	2.55	0.27	0.06	0.81
C: 32	2.85	3.09^a	1.98	2.07	2.85
D: 35	6.42	5.85	8.82	14.22^a	0.00
E: 29	1.29	1.89	3.63^a	1.89	-[b]

[a]Highest value measured for each male.

[b]Dead before termination of regeneration time.

From these data (Tables 2, 3, and 4) we see that each male follows a particular response in reproductive quality. By analyzing the changes in sperm count, spermatophore weight, and abnormalities, no common pattern was found; whereas male C shows a clear

increment in spermatophore weight in successive regenerations, male A presents a decline of the same parameter. The other males exhibit irregular fluctuations.

One interesting finding was that changes in spermatophore weight for a given male are not an indicator of sperm density within spermatophores. Only male E had a higher sperm count as the spermatophore weight increased.

Domesticated males, as for our pond-grown laboratory postlarvae, show an encouraging reproductive status. Males A, C, D, and E produced every new compound spermatophore with a sperm count similar to or better than previous regenerations. Male D improved from 6.42 millions at first regeneration to 14.22 millions at forth regeneration.

Table 3. Spermatophore weights (g) for five regenerations of individually marked *Penaeus stylirostris*.

Male:	Regeneration number				
Body weight (g)	1	2	3	4	5
A: 37	0.06[a]	0.04	0.04	0.03	0.03
B: 35	0.08[a]	0.05	0.07	0.08[a]	0.04
C: 32	0.03	0.04	0.04	0.05	0.07
D: 35	0.09	0.08	0.10[a]	0.08	0.00
E: 29	0.03	0.04	0.06[a]	0.04	–[b]

[a]Highest value measured for each male.

[b]Dead before termination of regeneration time.

Table 4. Abnormal sperm (%) for five spermatophore regenerations of individually marked *Penaeus stylirostris*. spn = Spermatophores were not regenerated.

Male:	Regeneration number				
Body weight (g)	1	2	3	4	5
A: 37	56.6	88.2	60.0	31.3	25.7[a]
B: 35	62.2	73.7	90.0	81.8	43.1[a]
C: 32	45.1	56.1	61.8	62.4	31.2[a]
D: 35	33.6	39.9	36.1	19.2[a]	spn
E: 29	64.3	33.5	35.2	31.3[a]	–[b]

[a]Lowest value measured for each male.

[b]Dead before termination of regeneration time.

Successive regenerations also had a positive effect over sperm abnormalities in every experimental male. Regardless of fluctuations in spermatophore weight and sperm count, each male improved its proportion of normal sperm by the forth or fifth regeneration.

Discussion

The observed increase in sperm count and the reduction in abnormalities in relation to size appear to be features of the maturation process in male penaeid shrimp. Young males of *P. monodon* (Motoh, 1981) and *P. vannamei* (Leung-Trujillo and Lawrence, 1985) have also been observed with high levels of abnormalities.

The maturation process in *P. stylirostris*, as documented by the regression equation, involves a progressive intensification of spermiogenesis: mitotic and meiotic division (King, 1948). Testes produce higher numbers of spermatids as the organism grows; nevertheless, the high frequency of abnormalities found in younger animals seems to indicate that the vas deferens, which is the place for final sperm maturation by completion of the subacrosome: spike formation (Shigekawa and Clark, 1986), does not mature at the same time that the testis begin to produce sperm. In this study, the minimum shrimp size showing immature spermatophores was 23.6 g.

With the purpose of understanding male maturation, some other experimental observations are pertinent. Wild *P. stylirostris* (mean weight = 49.4 g) used as brood stock for 5 weeks presented low levels of abnormalities (15.0%), yet sperm counts were very low (7.5 millions; unpublished data). However, wild *P. setiferus* (mean weight = 40 g) brought into captivity presented deteriorated spermatophores with no sperm at 35 days post-capture. The vas deferens at day 47 showed high sperm counts (263 millions/both vas deferens) and increased abnormalities (61.0%) compared to the spermatophore condition at collection (sperm count = 45 millions; abnormalities = 23%; Alfaro 1990).

By analyzing the previous information, we propose that male maturation has at least three independent levels. The first is testes maturation, which produces immature sperm. The second is vas deferens maturation, which completes sperm maturation by spike formation. The third level is spermatophore synthesis within ampoules, where the final product is packed. It is suggested that factors like stage of development, environment, and nutrition may affect the final quality of spermatophores by unknown mechanisms, which act at one or more of these levels.

This hypothesis would explain the higher abnormalities observed in younger males; it also would explain how mature males produce spermatophores with low abnormalities and low sperm count, and how spermatophore synthesis can be completely stopped in males presenting high number of sperm within vas deferens. The improvement in number of normal sperm independently of spermatophore weight and sperm count in

successive regenerations appears to support this hypothesis. Ejaculation and regeneration, under our experimental conditions, had a positive effect over vas deferens performance.

The finding about spermatophore weight and its quality is very important for commercial maturation units because it means that evaluating spermatophores for size and appearance does not yield information about sperm count. This was corroborated in a commercial maturation unit, where we found that wild *P. stylirostris* brood stock spermatophores with an excellent external appearance and weight (mean value = 0.16 g) had sperm counts of only 7.5 million.

The culture conditions under which we evaluated our young group (mean weight = 33.5 g) were not the optimum for penaeid shrimp maturation; nevertheless, the way they responded sexually, even without the enhancing influence of females, appears to be an indicator of the excellent reproductive qualities of domesticated and selected males.

Male reproductive tract degenerative syndrome (MRTDS) and male reproductive system melanization (MRSM), as they occur in captive *P. setiferus* (Alfaro, 1990), did not develop in our experimental group. This could be the result of species-specific defense responses, the degree of physiological adaptation to captivity, or to ejaculatory procedures.

Melanization has been reported as a problem for *P. stylirostris*, where Chamberlain et al. (1983) found a higher incidence of melanization in manually ejaculated shrimp. Harris and Sandifer (1986) also reported melanization of reproductive tissues in electrically ejaculated *Macrobrachium rosenbergii*. The lack of melanization in this study indicates that the protocol followed for artificial ejaculation may have an effect on the incidence of melanization. By ejaculating with gentle pressure to avoid damage to the gonopore and by using disinfected tweezers for final extraction, reproductive tract melanization in penaeid shrimp brood stock can be eliminated.

Acknowledgments

The author is grateful for the support of the Aquaculture Program: UNA-JICA, Escuela de Ciencias Biológicas, and Vicerrectoría de Investigación. I wish to thank Mr. F. Vives, production manager of Criadero de Camarones de Chomes S.A., for his permanent collaboration with this research.

References

Alfaro, J., 1990. A contribution to the understanding and control of the male reproductive system melanization disease of brood stock *Penaeus setiferus*. Master's thesis. Texas A&M University, College Station, Texas, USA.

Bray, W. A., Leung-Trujillo, J., Lawrence, A. L., 1985. Preliminary investigation on the effects of temperature, bacterial inoculation, and EDTA on sperm quality in captive *Penaeus setiferus*. J. World Maric. Soc. 16, 250 – 257.

Cavanaugh, G. M. (Ed.), 1956. Formulae and methods of the marine biological laboratory chemical room. Marine Biological Laboratory, Woods Hole, Massachusetts, USA.

Chamberlain, G.W., 1988. Stepwise investigation of environmental and nutritional requirements for reproduction of penaeid shrimp. PhD Thesis, Department of Wildlife and Fisheries Sciences, Texas A&M University, USA.

Chamberlain, G.W., Johnson, J. K., Lewis, D. H., 1983. Swelling and melanization of the male reproductive system of captive adult penaeid shrimp. J. World Maric. Soc. 14, 135 - 136.

Harris, S., Sandifer, P. 1986. Sperm production and the effects of electrically induced spermatophore expulsion in the prawn *Macrobrachium rosenbergii* (De Man). J. Crustacean Biol. 6 (4), 633 - 647.

King, J. E., 1948. A study of the reproductive organs of the common marine shrimp, *Penaeus setiferus* (Linnaeus). Biol. Bull. (Woods Hole) 94, 244 - 262.

Leung-Trujillo, J., Lawrence, A. L., 1985. The effect of eyestalk ablation on spermatophore and sperm quality in *Penaeus vannamei*. J. World Maric. Soc. 16, 258 - 266.

Leung-Trujillo, J., Lawrence, A. L., 1987a. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65, 363 - 370.

Leung-Trujillo, J., Lawrence, A. L., 1987b. Spermatophore regeneration times for three commercially important penaeid species: *Penaeus stylirostris*, *Penaeus vannamei*, and *Penaeus setiferus*. J. World Aquacult. Soc. 18 (1), 32A (Abstract 127).

Leung-Trujillo, J., Lawrence, A. L., 1988. The effect of ascorbic acid on sperm and spermatophore quality in *Penaeus vannamei* males fed prepared diets. J. World Aquacult. Soc. 19, 46A (Abstract 170).

Makinouchi, S., Primavera, J. H., 1987. Maturation and spawning of *Penaeus indicus* using different ablation methods. Aquaculture 62, 73 - 81.

Motoh, H., 1981. Studies on the fisheries biology of the giant tiger prawn, *Penaeus monodon*, in the Philippines. Technical paper No. 7, Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan, Iloilo, Philippines.

Ott, L., 1984. An introduction to statistical methods and data analysis. Duxbury Press, Boston, Massachusetts, USA, 775 pp.

Primavera, J.H., 1985. A review of maturation and reproduction in closed thelycum penaeids. In: Taki, Y., Primavera, J. H. and Llobrera, J. A. (Eds.), Proc. 1st Int. Conf. on the Culture of Penaeid Prawns/Shrimps, Aquaculture Department, SEAFDEC, Iloilo, Philippines, pp. 47-64

Shigekawa, K., Clark, W. H., 1986. Spermiogenesis in the marine shrimp, *Sicyonia ingentis*. Dev. Growth Differ. 28 (2), 95 - 112.

Talbot, P., Howard, D., Leung-Trujillo, J., Lee, T. W., Li, W. Y., Ro, H., Lawrence, A. L., 1989. Characterization of male reproductive tract degenerative syndrome in captive penaeid shrimp (*Penaeus setiferus*). Aquaculture 78, 365 - 377.

Chapter 5

Development and Deterioration of Spermatophores in Pond-Reared *Penaeus vannamei*

J. Alfaro and X. Lozano

Escuela de Ciencias Biológicas, Universidad Nacional,
Heredia, Costa Rica

Published in: J. World Aquacult. Soc. (1993), 24 (4): 522 – 529

Abstract

Spermatophore deterioration in pond-reared *Penaeus vannamei* was studied, and spermatophore quality was evaluated and improved. Sperm production of males collected from grow-out ponds was increased by two techniques: eyestalk ablation using a fresh frozen maturation diet (13% body weight); and laboratory culture using a diet of the formulated feed Nicovita Plus (3% body weight/ d) and frozen squid (2%). Findings are complementary to previous reports that eyestalk ablation improves quality of spermatophores in young (25.7 g) males. The timing of eyestalk ablation for activation of the endocrine mechanism, leading to improved spermatophore quality was also explored. After eyestalk ablation, performed between postmolt and intermolt stages, 26 g males required a minimum of three spermatophore regenerations or 42 d to significantly increase spermatophore size and sperm count. On the other hand, the laboratory culture (2.5 mo) technique improved the quality of spermatophores in successive regenerations for non-ablated males. In the present study, subadult *P. vannamei* produced spermatophores which, if not transferred or manually ejaculated, gradually deteriorated (successive stages are described), while a new compound spermatophore was being synthesized.

Introduction

Penaeid shrimp aquaculture has become a major industry in Asia and Latin America. This industry is based mainly on wild postlarvae or postlarvae produced in the laboratory from wild broodstock. Nevertheless, some companies are also using pond-reared animals as broodstock.

If shrimp farming is to be improved, one major concern is domestication as accomplished with major animal and plant species in land-based agriculture. Domestication, selection, and therefore improvement of culture animals in production characteristics are steps that will have to arise sooner or later in the industry.

It has been stated that wild broodstock perform better than reared broodstock. Egg production of laboratory matured *Penaeus californiensis* was inferior to mature females from the natural environment (Moore et al., 1974); however, proper nutrition can improve quality of spawnings (Emmerson, 1980). On the other hand, the eyestalk ablation technique, which is applied to wild and pond-reared females, affects the reproductive performance by causing ablated females to release fewer eggs per spawn and remature faster (Tan-Fermin, 1991). Finally, broodstock from closed life cycles has a commercial benefit over wild broodstock because of a better availability and predictability.

Closing the life cycle of penaeid shrimp was a first step for a new technological status of the field (Aquacop, 1979). Some research has evaluated maturation, spawning, and nauplii production of pond-grown broodstock (Chamberlain and Lawrence, 198; Chamberlain and Gervais, 1984). Wyban et al. (1990) showed that selection of *P. vannamei* females based on their reproductive performance can improve the quality and quantity of spawnings in future generations. In contrast to previous reports, it has also been demonstrated that pond-grown females can be utilized as broodstock for a long period of time (7 mo) after eyestalk ablation (Wyban et al., 1987).

It is thought that reproductive performance of pond-grown *P. vannamei* males has not been studied in depth. Research on spermatophore quality in penaeid shrimp is fragmentary. The first documentation of the effects of eyestalk ablation on spermatophore condition and evaluation of sperm quality in penaeid shrimp were provided by Leung-Trujillo and Lawrence (1985, 1987), respectively. Posteriorly, practical benefits of male eyestalk ablation have been reported. Male *P. vannamei* reuse was improved by eyestalk ablation (Salvador et al., 1988), and nauplii production of *P. stylirostris* was increased by artificial insemination using ablated males (Ottogalli, 1989).

Blackening of reproductive tracts and deterioration of spermatophores have been partially studied in penaeid shrimp. Both conditions have been considered as different stages of the same problem (Bray et al., 1985; Leung-Trujillo and Lawrence, 1987; Talbot et al., 1989). However, Alfaro (1990) found no relationship between both conditions and established that the primary cause of a male reproductive system melanization (MRSM) disease of captive *P. setiferus* was a bacterial infection. Dougherty and Dougherty (1989) studied the electron microscopy and histochemistry of *P. vannamei* melanized spermatophores and found no bacteria associated with the condition.

The deterioration of spermatophores in captive *P. setiferus* is a predictable event (Leung-Trujillo and Lawrence, 1987). The process involves not only the spermatophores but also the entire reproductive tract (Talbot et al., 1989) producing sterile males in a short period of time (1 mo). Talbot et al. (1989) suggested that bacterial infection was not the primary cause of this problem; moreover, Alfaro (1990) found no evidences of bacterial infection associated with onset of the condition, and suggested stress as a possible cause of the syndrome. Observations on the onset of spermatophore deterioration in other penaeid shrimp are required.

The objectives of this study were: 1) to further improve the basic knowledge about male eyestalk ablation by applying a different experimental protocol; 2) to evaluate the effect of laboratory culture on spermatophore quality of young pond-

grown *P. vannamei*; and 3) to study the process of spermatophore deterioration in this species.

Materials and methods

Animals

P. vannamei were selected during harvesting of commercial semi-intensive earthen ponds at Criadero de Camarones de Chomes S.A., Costa Rica. Laboratory postlarvae were stocked in nursery ponds at 150 m^{-2} for 22 d and then transferred to grow-out ponds with supplementary aeration (2 hp ha^{-1}) at 13.2 shrimp m^{-2} . Animals were fed with Nicovita feeds for 110 d, and the average growth rate was 1.2 g wk^{-1}.

Male Eyestalk Ablation Experiment

An experimental group was used to evaluate the effect of eyestalk ablation on spermatophore quality. Before initiation of treatments, males were analyzed for molt stages using the technique described by Robertson et al. (1987). Male *P. vannamei* (mean weight = 25.7; standard error of the mean (SEM) = 2.4 g) were divided into two groups of four animals each, selecting only postmolt to intermolt stage shrimp. One group's eyestalks were unilaterally ablated by cauterization, and the other group was used as a control. Females were not stocked to eliminate this factor as an exogenous variable perhaps affecting spermatophore quality.

The experiment was conducted in a recirculating culture unit (1 m^2) with external biological filtration and automatic temperature control (Earth Shyokai Co., Ltd, Tokyo, Japan). Water was exchanged weekly at approximately 10-15% of the total volume. A fresh-frozen diet consisting of shrimp heads *P. occidentalis*, marine bloodworms *Nereis viridens*, giant squid *Dosidicus gigas*, and bivalves *Anadara tuberculosa* was provided at a ratio of 1:1:1:0.25, respectively, at 13% wet body weight per day. Each food item was given individually at different times: 09:00, 12:00, 14:00, and 17:00 h, respectively. Salinity was maintained at 30 ppt, temperature at 29 °C, and pH at 7.7-7.9. An acclimation period of 13 d was adopted before initiation of treatments.

Males were ejaculated before treatment (0 wk), and at 2, 4, and 6 wk until 3 regenerations were obtained. Ejaculation was performed following a modified technique of that described by King (1948), consisting of manually extruding a portion of the spermatophore with gentle pressure, and then employing fine tweezers to completely remove the unit. The quality of spermatophores was evaluated as described below.

Laboratory Culture Experiment

Growth of selected animals (N = 280; 1:1 female: male ratio) was continued in the laboratory, stocking them in two fiberglass tanks (15 m^2) at 9.3 shrimp m^{-2} . Water exchange was kept at 100% daily, using new water pretreated by high pressure silica sand filtration and sedimentation. Animals were fed two times a day at 3% of body weight with Nicovita Plus and 2% of body weight with fresh-frozen squid. Salinity was 30 ± 2 ppt and temperature 28 ± 1 °C during the course of the experiment.

The experimental population was randomly sampled (N = 20) once a month for total weight, to the nearest 0.1 g, and evaluation of spermatophore condition. Additionally, two groups of males (N1 = 5, N2 = 5) were individually marked by a combined technique of eyestalk banding and uropod cutting; these males were ejaculated every 2 wk, and spermatophore quality was analyzed. Another group (N = 6) was also marked, and individual spermatophore condition was followed over time. This group was not ejaculated.

Spermatophore Quality Analysis

The parameters measured were compound spermatophore weight, to the nearest 0.01 g, sperm count per compound spermatophore, and percentage of abnormal sperm.

Sperm counts were performed by homogenizing the compound spermatophore in a glass tissue grinder, containing 3.0 ml of artificial seawater, prepared according to Cavanaugh (1956). The suspension was then mixed several times to insure homogeneity, and three samples were counted using a hemacytometer; the sample volume was 0.0001 ml.

Percentage of abnormal sperm was obtained by recording, over a transect of a microscope slide, number of normal sperm (spherical body and elongate spike) (Talbot et al., 1989) and number of abnormal sperm (malformed bodies and bent or missing spikes). At least 100 cells for one of the categories were recorded.

Data Analysis

Eyestalk ablation experiment. Data on spermatophore weight and sperm count per sample volume (0.0001 ml) were statistically analyzed with a modified *t* test for independent samples. Percentage of abnormalities was analyzed with a modified *t* test for unequal variance at $\alpha = 0.01$ (Ott, 1984). Sperm count and percentage abnormality data were transformed, to make the variance independent of the mean, by square root of Y + 3/8 (Bray et al., 1985) and arcsine (Leung-Trujillo and Lawrence, 1985), respectively.

Laboratory culture experiment. Data on spermatophore analysis are presented as mean values ± SEM.

Results

A sample of *P. vannamei* collected from grow-out ponds at harvesting showed the reproductive quality parameters presented in Table 1. These young males are of very low quality as broodstock; nevertheless, the quality of spermatophores produced was improved as shown in Tables 2, 3 and 4.

An evaluation of the effect of eyestalk ablation on individuals is presented in Tables 2 and 3. It is interesting to note that in the first and second spermatophore regenerations, the changes in sperm count based on treatment are not clear; nevertheless, control shrimp present very low sperm counts in each regenerated spermatophore. It is in the third regeneration that eyestalk ablated males show a visible improvement over the control group. The improvement effect of eyestalk ablation after

Table 1. Baseline spermatophore data for *Penaeus vannamei* grown in semi-intensive earthen ponds. Data are presented as mean ± SEM.

Group[a]	Body weight (g)	Sample size (N)	Spermatophore weight (g)	Sperm count (millions)	Abnormal sperm (%)
A	25.7 ± 2.4	6	0.01 ± 0.00	1.87 ± 2.36	85.7 ± 21.3
B	22.2 ± 1.5	12	0.01 ± 0.004	1.48 ± 3.47	52.0 ± 8.9

[a] Groups A and B were collected in October 1990 and August 1991, respectively.

Table 2. Sperm count (millions) in successive spermatophore regenerations[a] for eyestalk ablated *Penaeus vannamei.*

Replicate/ treatment	Initial body weight (g)	Spermatophore regeneration		
		1 (2 wk)	2 (4 wk)	3 (6 wk)
Ablated 1	28.2	6.07	11.71	21.53
Ablated 2	25.7	0.00	2.17	10.83
Ablated 3	28.6	6.43	3.84	12.04
Ablated 4	24.8	4.99	0.00	6.75
Control 1	23.7	0.00	0.00	0.00
Control 2	24.6	0.54	1.86	4.63
Control 3	25.1	0.05	0.12	0.09
Control 4	24.1	0.10	1.10	2.92

[a] Spermatophores analyzed were normal and white.

Table 3. Mean values[1] of spermatophore quality for eyestalk ablated *Penaeus vannamei* at regeneration 3.

	N	Spermatophore weight (g)	Sperm count (millions)	Abnormal sperm (%)
Baseline	6	0.01 ± 0.00	1.87 ± 2.36	85.7 ± 21.3
Eyestalk ablation	4	0.03^{A} ± 0.005	12.79^{A} ± 6.25	41.1^{A} ± 12.8
Control	4	0.01^{B} ± 0.00	1.91^{B} ± 2.26	51.7^{A} ± 42.2

[1] Means with the same letters are not statistically different ($P < 0.01$).

Table 4. Spermatophore weight, number of sperm, and sperm abnormalities in successive spermatophore regenerations for laboratory cultured *Penaeus vannamei*[a]. Data are presented as mean ± SEM.

	Spermatophore regeneration		
Condition	1[b] (0 wk)	2 (2 wk)	3 (4 wk)
N	10[c]	10	10
Spermatophore weight (g)	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
Sperm count (millions)	19.78 ± 4.72	18.73 ± 5.69	16.76 ± 7.77
Sperm abnormality (%)	33.1 ± 26.6	32.1 ± 22.2	39.4 ± 29.3

[a] Baseline values are presented in Table 1.

[b] First analysis performed at 2.5 mo from pond harvesting.

[c] Mean body weight = 24.2 ± 2.6 g.

three spermatophore regenerations was statistically significant ($P < 0.01$) in spermatophore size and sperm count. Nevertheless, percentages of abnormal sperm were not statistically different ($P > 0.01$).

Table 4 summarizes pooled data on spermatophore quality for successive spermatophore regenerations of two groups of tagged males (N1 + N2 = 10) taken from a laboratory cultured male population of 140 *P. vannamei* (sample size is 7% of male population). Fig. 1 summarizes observations of external appearance of spermatophores within ampoules in randomly sampled non-ejaculated males. This graphic representation indicates that different stages of deterioration appeared with time, and because these males did not mate and were not ejaculated, the proportion of normal spermatophores decreased. The cases showing empty ampoules registered on September 30 are explained by the fact that on September 25, some shrimp were ejaculated. The first cases of visible deterioration were found after two months of

laboratory culture, but it was not until the third month that completely brown spermatophores were observed. Complementary to the previous observations, a group of males were tagged to allow following spermatophore stages over time (Table 5). These observations indicate that darkening of spermatophores is very slow; males 3 and 5 did not change in color during 6 and 7 wk, respectively. On the other hand, males 1 and 2 developed a partially brown coloration after 8 and 6 wk, respectively. Male 4 always presented a partially brown coloration during 6 wk of culture, and male 6 changed from partially brown to completely brown in 4 wk.

Based on these observations, the following stages of spermatophore condition were defined:

Stage I: normal, white spermatophores.

Stage II: partially brown spermatophores.

Stage III: completely brown spermatophores.

Stage IV: brown and compacted residue.

Stage V: dark brown reduced residue.

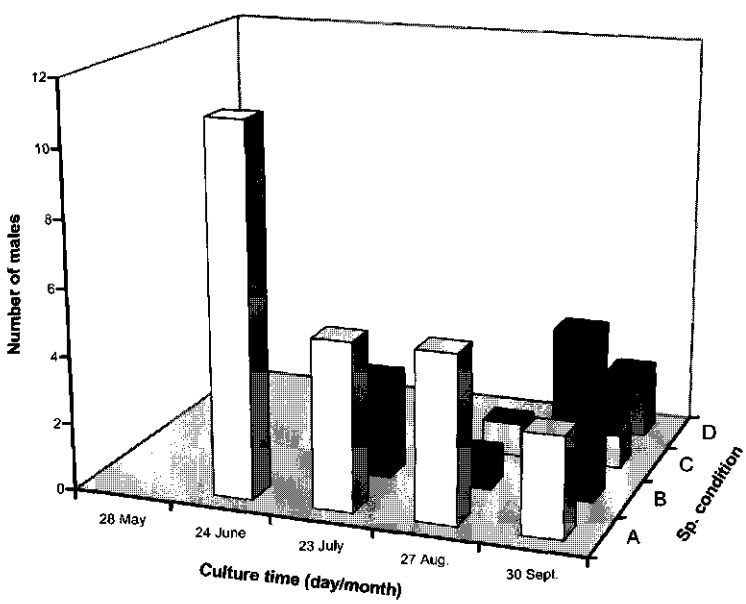

Figure 1. Spermatophore condition-frequency distribution over time for a laboratory-cultured *Penaeus vannamei* population (N= 280; n= 20; sex ratio = 1:1). Symbols are: A, normal white spermatophores; B, partially brown spermatophore; C, completely brown spermatophore; D, empty ampoule.

It was observed by manual ejaculation that males in stages IV and V can release stage I spermatophores behind the deteriorated one. Moreover, during the experimental period (7 mo) only two cases of black reproductive systems were found. These animals presented black ampoules and dark brown distal and medial vas deferens. The black coloration of ampoules was associated with the spermatophores, and the appearance and intensity of the dark coloration of these spermatophores were different from deteriorated spermatophores.

Contrary to what has been observed in non-ejaculated males, the ejaculated group always presented spermatophores in stage I. The first samples were taken from males exhibiting various degrees of deterioration, and in successive regenerations every male presented white spermatophores, except for some animals with forming spermatophores.

Table 5. Spermatophore condition[a] over time in individually marked *Penaeus vannamei* held under laboratory conditions following pond culture.

	Male					
Week	1	2	3	4	5	6
0	N	N	N	PB	N	PB
2	F-N				N	
3		EA	N	PB		PB
4		F-N	N	PB		CB
5	N	EA	N	PB	N	CB
6	N	PB	N	PB	N	CB
7	N				N	
8	PB					

[a] Spermatophore condition is evaluated based on the following code: N = normal spermatophore, F = spermatophore in formation, PB = partially brown spermatophore, CB = completely brown spermatophore, EA = empty ampoule.

Discussion

Sperm production in pond grown *Penaeus vannamei* can be improved by eyestalk ablation and laboratory culture. The first technique, which incorporated eyestalk ablation, was demonstrated by Leung-Trujillo and Lawrence (1985) following a different protocol. Their study utilized wild juveniles grown at low density in ponds to an initial experimental weight of 35.4 g, and stocking of females with males. The findings of the present study are complementary to those of the previous authors, since it has been demonstrated that eyestalk ablation also improves the quality of

spermatophores in young males (body weight = 25.7 g) held without females. Additionally, after eyestalk ablation, performed between postmolt and intermolt stages, male *P. vannamei* required at least 3 spermatophore regenerations over a 42 d period to statistically improved their spermatophore quality in terms of size and sperm count.

Sperm abnormalities in eyestalk ablated animals did not decrease significantly during the experimental period. It seems that young males can be rapidly activated for spermatophore synthesis and spermatogenesis by eyestalk ablation, but complete spermatid maturation in the vas deferens (Shigekawa and Clark, 1986) is not affected at a similar rate. This high percentage of abnormalities in young males also has been observed in *P. stylirostris* (Alfaro, in press).

Laboratory culture techniques allowed improvement of the quality of spermatophores in young males; however, fertilization capacities were not evaluated. After 2.5 mo from harvesting, males (24.2 ± 2.6 g) were producing better spermatophores, with higher numbers of sperm and lower percentage of abnormalities. From the study of Leung-Trujillo and Lawrence (1985), it can be calculated that their control males (body weight = 35 g) at initiation presented low sperm counts (5.61 million), and high abnormalities (77.7%). After 104 d of culture males produced spermatophores with the following characteristics: weight = 0.045, sperm count = 32 million, and sperm abnormalities = 10%. Sperm counts in the present study are comparable to those of the previous authors, but sperm abnormalities are higher (Table 4).

Nutritional requirements for spermatogenesis and spermatophore synthesis appear to be met with the experimental diet utilized in this study, judging by increase spermatophore weights and higher sperm counts over time. The high percentage of abnormalities does not seem to be related to a nutritional factor because other studies, using the same diet and male size (unpublished data), gave low sperm abnormalities.

These observations on spermatophore deterioration indicate that *P. vannamei* produce spermatophores which, if not transferred to females or manually ejaculated, gradually deteriorate passing through the stages that were described. Therefore, this deterioration process appears common in the laboratory environment; whether this process also occurs in nature or in other maturation systems has to be investigated. Once a compound spermatophore is produced, it will be useful for a given period of time. After that period it may deteriorate until a residue is formed, while a new compound spermatophore is being produced. Aquacop (1983) observed *P. vannamei* males with small or "brown spermatophores with necrosis," suggesting poor sperm quality was responsible for low percentage fertilization. On the other hand, Leung-Trujillo and Lawrence (1985) found, at the end of 104 d with water temperatures as low as 17.8 °C, only well-formed spermatophores with no evidence of any discoloration or

deterioration. It is possible that low temperature retarded the normal process of deterioration and that mating renewed spermatophores. In *P. setiferus*, Bray et al. (1985) found that low temperatures retarded the decline in sperm quality.

Dougherty and Dougherty (1989) found that black spermatophores of *P. vannamei* presented melanin droplets not only at the spermatophore surface, but also in the sperm mass matrix. The authors did not find bacterial invasion and suggested that hemocytic or nonhemocytic enzymes could be involved in the pathological melanization, which results under certain abnormal conditions. Observations on different penaeid species indicate that spermatophores may develop light or strong pigmentation, and it seems that the mechanisms that trigger the condition could be of different origin, even bacterial infection as established in *P. setiferus* (Alfaro, 1990).

Pond-grow *P. vannamei* deteriorates slowly (2-3 mo at tropical temperatures), and either by manual ejaculation or naturally, new spermatophores are regenerated. It is proposed that the spermatophore deterioration, observed in *P. vannamei*, is a normal process in penaeid shrimp reproduction.

Acknowledgments

We thank the programs UNA-JICA and UNA-LUW for their financial support; Criadero de Camarones de Chomes S.A., and specially Ing. Eduardo Velarde, General Manager, and Mr. Fernando Vives, Production Manager, for their constant cooperation; and Bill Bray (Texas A&M Shrimp Mariculture Project) for his comments on the manuscript.

References

Alfaro, J.,1990. A contribution to the understanding and control of the male reproductive system melanization disease of brood stock *Penaeus setiferus*. Master's thesis. Texas A&M University, College Station, Texas, USA.

Alfaro, J., in press. Reproductive quality evaluation of male *Penaeus stylirostris* from a grow-out pond. J. World Aquacult. Soc.

Aquacop, 1979. Penaeid reared broodstock: closing the cycle of *P. monodon*, *P. stylirostris* and *P. vannamei*. Proc. World Maric. Soc. 10, 445 – 452.

Aquacop, 1983. Constitution of broodstock, maturation, spawning, and hatching systems for penaeid shrimp in the Centre Oceanologique du Pacifique. In: McVey, J. P., (Ed.), CRC Handbook of Mariculture, volume 1: Crustacean aquaculture. CRC Press, Boca Raton, Florida, USA, pp. 105 – 121.

Bray, W. A., Leung-Trujillo, J., Lawrence, A. L., 1985. Preliminary investigation on the effects of temperature, bacterial inoculation, and EDTA on sperm qualtity in captive *Penaeus setiferus*. J. World Maric. Soc. 16, 250 - 257.

Cavanaugh, G. M. (Ed.), 1956. Formulae and methods of the marine biological laboratory chemical room. Marine Biological Laboratory, Woods Hole, Massachusetts, USA.

Chamberlain, G. W., Gervais, N. F., 1984. Comparison of unilateral eyestalk ablation with environmental control for ovarian maturation of *Penaeus stylirostris*. J. World Maric. Soc. 15, 29 - 30.

Chamberlain, G. W., Lawrence, A. L., 1981. Effect of light intensity and male and female eyestalk ablation on reproduction of *Penaeus stylirostris* and *P. vannamei*. J. World Maric. Soc. 12 (2), 357 - 372.

Dougherty, W. J., Dougherty, M. M., 1989. Electron microscopical and histochemical observations on melanized sperm and spermatophores of pond-cultured shrimp, *Penaeus vannamei*. J. Invertebr. Pathol. 54, 331 - 343.

Emmerson, W. D., 1980. Induced maturation of prawn *Penaeus indicus*. Mar. Ecol. Prog. Ser. 2, 121 - 131.

King, J. E., 1948. A study of the reproductive organs of the common marine shrimp, *Penaeus setiferus* (Linnaeus). Biol. Bull. (Woods Hole) 94, 244 - 262.

Leung-Trujillo, J., Lawrence, A. L., 1985. The effect of eyestalk ablation on spermatophore and sperm quality in *Penaeus vannamei*. J. World Maric. Soc. 16, 258 - 266.

Leung-Trujillo, J., Lawrence, A. L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65, 363 - 370.

Moore, D. W., Sherry, R. W., Montanez, F., 1974. Maturation of *Penaeus californiensis* in captivity. Proc. World Maric. Soc. 5, 445 - 449.

Ott, L., 1984. An introduction to statistical methods and data analysis. Duxbury Press, Boston, Massachusetts, USA.

Ottogalli, L. ,1989. Increased production of nauplii (*Penaeus stylirostris* Stimpson, Mexican strain) from a captive broodstock, using ablated and regenerated males for artificial insemination. J. World Aquacult. Soc. 20 (1), 61A.

Robertson, L., Bray, W., Leung-Trujillo, J., Lawrence, A. L., 1987. Practical molt staging of *Penaeus setiferus* and *Penaeus stylirostris*. J. World Aquacult. Soc. 18 (3), 180 - 185.

Salvador, J. A., Jaramillo, B., Romero, Z., Lucien-Brun, H., Lee, D. O'C., 1988. A comparative analysis of sperm quality, between wild caught males and unilaterally eyestalk ablated regenerated males in a commercial *Penaeus vannamei* facility. J. World Aquacult. Soc. 19 (1), 61A.

Shigekawa, K., Clark, W. H., 1986. Spermiogenesis in the marine shrimp, *Sicyonia ingentis*. Dev. Growth Differ. 28 (2), 95 - 112.

Talbot, P., Howard, D., Leung-Trujillo, J., Lee, T. W., Li, W. Y., Ro, H., Lawrence, A. L., 1989. Characterization of male reproductive tract degenerative syndrome in captive penaeid shrimp (*Penaeus setiferus*). Aquaculture 78, 365 - 377.

Tan-Fermin, J. D., 1991. Effects of unilateral eyestalk ablation on ovarian histology and oocyte size frequency of wild and pond-reared *Penaeus monodon* (Fabricius) broodstock. Aquaculture 93, 77 - 86.

Wyban, J. A., Cheng, S. L., Sweeney, J. N., Richards, W. K., 1987. Observations on development of a maturation system for *Penaeus vannamei*. J. World Aquacult. Soc. 18 (3), 198 - 200.

Wyban, J. A., Oyawa, R., Deupree, R., Chang, C., Sweeney, J., 1990. Selective breeding for reproductive quality in marine shrimp. World Aquaculture 90, 10-14 June 1990, Halifax, Nova Scotia, Canada.

Chapter 6

Effect of 17-Alpha-Methyltestosterone and 17-Alpha-Hydroxyprogesterone on the Quality of White Shrimp, *Penaeus vannamei* Spermatophores

J. Alfaro

Escuela de Ciencias Biológicas, Universidad Nacional,
Heredia, Costa Rica

Published in: J. World Aquacult. Soc. (1996), 27 (4): 487 – 492

Abstract

Controlled reproduction of penaeid shrimp requires a better utilization of males by sperm quality monitoring and sperm quality improvement. Spermatophores of white shrimp, *Penaeus vannamei* were improved, in terms of increased sperm count, spermatophore weight, and a reduced incidence of sperm abnormalities by a single injection of 17-alpha-methyltestosterone at 0.01 or 0.1 μg g^{-1} body weight. 17-alpha-hydroxyprogesterone did not induce a significant improvement in spermatophore quality. These findings indicate that a steroid injection program should be evaluated as a practical way of improving spermatophore quality in commercial operations.

Introduction

Spermatophores of penaeid shrimp may vary considerably in quality and have been evaluated in terms of spermatophore weight, sperm count, and the percentage incidence of abnormalities (Leung-Trujillo and Lawrence, 1985; Leung-Trujillo and Lawrence, 1987). More recently the activation of an acrosome reaction by the egg water technique developed by Griffin et al. (1987) has been evaluated as a more accurate measure of sperm quality (Pratoomchat et al., 1993; Wang et al., 1995).

Unilateral eyestalk ablation (Leung-Trujillo and Lawrence, 1985) as well as adequate husbandry in the laboratory (Alfaro and Lozano, 1993) are reported to increase spermatophore weight and sperm counts, and reduce sperm abnormalities. However, Browdy (1992) found no significant effect in mating frequency, spawn size, fertilization or hatch for females mated with ablated *Penaeus setiferus*. Ottogalli et al. (1988) reported no significant increase in fertilization rates or numbers of natural matings using ablated *P. stylirostris*, and Pratoomchat et al. (1993) found no increase in spermatophore weight and sperm quality for ablated *P. monodon*. Blackening and deterioration of spermatophores of *P. setiferus* (Leung-Trujillo and Lawrence, 1987; Chamberlain, 1988; Talbot et al., 1989; Alfaro, 1990) indicate poor spermatophore quality, and reduced reproductive capacity. The blackening of spermatophores is a necrosis of the chitinous envelope and sperm mass matrix, associated with melanin deposition (Dougherty and Dougherty, 1989).

The role of the androgenic gland of crustaceans was first established in the amphipod *Orchestia gammarella* and some years later other crustaceans were evaluated (Charniaux-Cotton et al., 1966). The gland is the endocrine organ which determines primary and secondary male sexual characteristics (Charniaux-Cotton, 1960; Fingerman, 1987; Charniaux-Cotton and Payen, 1988). The first report of the isolation of an androgenic gland hormone (AGH) was done by Hasegawa et al. (1987). They isolated two peptides, AGH I and AGH II which induced primary and secondary male

sexual characteristics in *Armadillidium vulgare*. It seems that other androgenic gland extracts like farnesylacetone and steroids play complementary roles to AGH; some steroids have been identified from androgenic glands (AG) like conversion of androstendione to testosterone by *Homarus americanus* AG and conversion of progesterone to hydroxyprogesterone, androstendione, testosterone and deoxycorticosterone by *Callinectes sapidus* AG (Sagi, 1988).

Sex reversal in crustaceans has been accomplished by surgical removal or implantation of androgenic glands; in *Macrobrachium rosenbergii* the bilateral androgenic gland ablation at early developmental stages generates complete feminization (Nagamine et al., 1980a). Complementary, masculinization is induced by androgenic gland implantation regardless of developmental stage (Nagamine et al., 1980b). However, there have been few studies of this gland in decapod crustaceans (Huquet and Huquet, 1971; Taketomi et al., 1990); for penaeid shrimp the only known references of the anatomical location of this gland was provided by Payen et al. (1982) and Alfaro (1994).

Since it has been demonstrated that spermatophore quality can be improved by eyestalk ablation in young *P. vannamei* (Alfaro and Lozano, 1993), this investigation was designed to evaluate the effect on spermatophore quality of a single injection of 17-alpha-methyltestosterone or 17-alpha-hydroxyprogesterone into young males of *P. vannamei*.

Materials and methods

Experimental design

Healthy male *P. vannamei* were selected based on size, general appearance, and presence of spermatophores from earthen ponds at harvesting after 90-100 days of semi-intensive culture at Criadero de Camarones de Chomes S.A. Two independent experiments were carried out. Molt stages were not monitored since spermatophore synthesis, but not spermatogenesis, is closely related to the intermolt cycle and the number of sperm in a new spermatophore remains constant throughout the intermolt cycle (Heitzman et al., 1993).

The first experiment was designed to evaluate the effect of a single injection of 17-alpha-methyltestosterone (Sigma Co.) into male shrimp. Males (19.9 ± 1.6 g) showing visually normal spermatophores were first acclimatized in an indoor circular tank for one month, feeding 3% with a commercial diet (Nicovita) per day. Then, 18 males were selected and transferred to one experimental tank (volume = 0.6 m^3), and fed 1.0-1.5% with the Nicovita diet and 3-4% frozen squid. One week acclimatization was adopted in order to reduce stress associated with handling and the laboratory environment. Water exchange was 100% daily through a continuous flow system.

In the second assay 17-alpha-hydroxyprogesterone (Sigma Co.) was evaluated. 15 males (mean body weight = 22.2 ± 1.5 g) collected from earthen ponds were acclimatized in one experimental tank (0.6 m^3) for one week. They were fed 1.0-1.5% with a Nicovita diet and 3-4% frozen squid per day. The only difference in the protocol between tests was the inclusion of a pre-acclimation step in the first experiment.

Each assay included three treatments; each steroid hormone was tested at 0.01 and 0.1 μg g^{-1} body weight. A control group was present in each experiment. Hormone doses and preparation protocol are based on Yano (1985) and modified for 0.04 - 0.05 ml injections. A concentrated stock was prepared the day before injection by dissolving the estimated amount of pure hormone (± 0.05 mg) up to 50 ml with 95% ethanol. Working solutions were prepared immediately before injection by diluting with sterile saline solution (0.85% NaCl), 1.0 and 0.1 ml of the concentrated hormone stock to obtain 25 ml of the two hormone doses (0.1 and 0.01 μg g^{-1} body weight, respectively). The control solution was prepared by diluting 1.0 ml of ethanol with saline solution to 25 ml. Animals were injected laterally in the third abdominal segment with 0.04 to 0.05 ml of the working solution based on individual body weight. There were six and five replicates per treatment for the first and second experiment, respectively. Replicates were individually tagged by uropod cutting.

Spermatophores were manually expelled from the males at 29 and 39 days (17-alpha-methyltestosterone), and 14 and 28 days (17-alpha-hydroxyprogesterone) after injection. Spermatophores were evaluated in terms of compound (two halves) spermatophore weight to the nearest 0.01 g, sperm count per compound spermatophore, and percentage of abnormal sperm, following protocols previously described (Alfaro, 1993). An arbitrary code was adopted to evaluate spermatophore appearance: a) normal spermatophores: both units showed white color and were easily expelled, b) blackening spermatophores: both units had partial or complete dark pigmentation, and c) spermatophores in formation: both units showed white color but could not be expelled.

Data analysis

Statistical comparison between treatments was performed using the replicates showing normal spermatophores. Animals which presented blackening spermatophores and spermatophores in formation within each treatment at the time of sampling were not included in the statistical analysis.

Data on spermatophore weight, sperm count, and sperm abnormalities were analyzed using One Way Classification Analysis of Variance (AOV) and Tukey's W procedure (Ott, 1984). Prior to analysis, data on sperm count and abnormalities were transformed using squared root of Y + 3/8 (Bray et al., 1985) and arcsine (Leung-Trujillo and Lawrence, 1985), respectively, to make the variance independent of the

mean. Additionally, comparisons between 2 means were analyzed using the t-test for independent samples with unequal variance (Ott, 1984). Data are presented as mean ± standard deviation.

Results

Survival was 100% for both hormone doses and 67% for control group for the 17-alpha-methyltestosterone experiment. At the time of sampling, replicates from each treatment presented three categories of spermatophores (Table 1), and the statistical comparison is performed with data from normal spermatophores (Tables 2). The incidence of blackening spermatophores was higher at 0.1 μg g^{-1} 17-alpha-methyltestosterone.

17-alpha-methyltestosterone improved the sperm count at 0.01 and 0.1 μg g^{-1} body weight in experimental males (Table 2; $P < 0.05$). No significant difference in sperm count occurred between the 0.01 and 0.1 μg g^{-1} body weight doses at either the first or the second assessment. There was a reduction in sperm count between the first and the second assessment in each treatment. Spermatophore weight (Table 2) increased as hormone dose increased ($P < 0.05$) at the first assessment. The second assessment gave no statistical differences in spermatophore weight between treatments ($P > 0.05$).

Table 1. Incidence of various spermatophore appearances[a] in the experiment injecting 17-alpha-methyltestosterone.

17-alpha-methyltestosterone dose (μg g^{-1})	Number of males					
	First ejaculation			Second ejaculation		
	N	B	F	N	B	F
0.10	3	3	0	3	2	1
0.01	3	2	1	4	1	1
Control[b]	3	1	0	4	0	0

[a] N: normal spermatophores; B: blackening spermatophores; F: spermatophores in formation. Survival rates were 100% for both hormone doses, and 67 % for control group.

[b] Control = 0.1 μl ethanol g^{-1} body weight in 50 μl of saline solution.

Sperm abnormality did not vary significantly between assessment periods (Table 2, analysis is not shown). The standard deviation was high for the control group in both assessments. However, comparison of the two assessments at each hormone dose (t-test) indicates that at 0.1 $\mu g\ g^{-1}$, percentages of abnormalities were similar ($P > 0.05$), but at 0.01 $\mu g\ g^{-1}$ there was a significant increase in the percentage of abnormalities at the second assessment ($P < 0.05$); for the control there was no significant increase in abnormalities ($P > 0.05$).

In the second experiment survival was 100% for each treatment. 17-alpha-hydroxyprogesterone did not significantly improve ($P > 0.05$) the sperm count at 0.01 and 0.1 $\mu g\ g^{-1}$ body weight in either assessment (Table 3, replicates with spermatophores in formation were not included). Spermatophore weights were very low (< 0.01 g), and sperm abnormalities were high but not analyzed statistically because some replicates in each treatment presented no sperm within spermatophores.

Discussion

This is the first report of a direct improvement of spermatophore quality, in terms of spermatophore weight, sperm count, and decreased sperm abnormalities by injecting an exogenous steroid hormone.

17-alpha-methyltestosterone induced an important improvement in spermatophore quality. Control males produced 11 million sperm per compound spermatophore, while by injecting a single dose of 0.1 and 0.01 $\mu g\ g^{-1}$ body weight, sperm counts increased 3 fold (32 million) in 20 g males. Moreover, a low percentage of sperm abnormalities were obtained. These values are comparable with those measured by Leung-Trujillo and Lawrence (1985) for 35 g unablated *P. vannamei* (spermatophore weight = 0.045 g, sperm count = 32 million, and sperm abnormalities = 10%).

Data obtained indicate that the enhancing effect measured in sperm count and spermatophore weight decreased in the second assessment (39 days after hormone injection). This could be the result of a reduction in hormone levels from body fluids since only one hormone injection was administered. However, sperm abnormalities at 0.1 $\mu g\ g^{-1}$ stayed low in the second assessment, but at 0.01 $\mu g\ g^{-1}$ abnormalities increased significantly ($P < 0.05$).

A higher incidence of blackening spermatophores was observed at 0.1 ug g^{-1} 17-alpha-methyltestosterone. This condition can be overcome by removing those deteriorated units, after which the ampoules may produce normal spermatophores (Alfaro and Lozano, 1993). Both doses gave similar results for sperm count, indicating that hormone levels evaluated were high. Lower levels of hormone could give similar

Table 2. Effects of 17-alpha-methyltestosterone on sperm quality in *Penaeus vannamei*.

	17-alpha-methyltestosterone dose[1] (μg g^{-1})		
	0.10	0.01	Control[2]
First ejaculation[3]			
N	3	3	3
Sperm count (*millions*)[4]	$31.70^{a} \pm 12.02$	$32.67^{a} \pm 7.53$	$10.93^{b} \pm 6.16$
Spermatophore weight (g)[4]	$0.04^{a} \pm 0.01$	$0.03^{ab} \pm 0.00$	$0.02^{b} \pm 0.01$
Sperm abnormalities (%)[5]	$24.7^{a} \pm 3.1$	$17.0^{a} \pm 6.7$	$38.4^{a} \pm 31.0$
Second ejaculation[6]			
N	3	3	3
Sperm count (millions)[4]	$19.00^{a} \pm 8.70$	$21.13^{a} \pm 2.94$	$4.07^{b} \pm 2.83$
Spermatophore weight (g)[4]	$0.02^{a} \pm 0.01$	$0.03^{a} \pm 0.00$	$0.02^{a} \pm 0.00$
Sperm abnormalities (%)[5]	$23.4^{a} \pm 9.7$	$35.5^{b} \pm 7.2$	$58.3^{a} \pm 20.7$

[1] Single injection per individual. Mean body weight = 19.9 ± 1.6 g.

[2] Control = 0.1 μl ethanol g^{-1} body weight in 50 μl of saline solution.

[3] 29 days after hormone injection.

[4] Different letters within row indicate statistically significant differences ($P < 0.05$).

[5] Different letters within column indicate statistically significant differences ($P < 0.05$)

[6] 39 d after hormone injection.

Table 3. Effects of 17-alpha-hydroxyprogesterone on sperm count in *Penaeus vannamei*. Same letters within column indicate no statistically significant differences ($P < 0.05$). Spermatophore weights were ≤ 0.01 g, and due to sperm counts of absolute zero for each treatment, sperm abnormalities were not statistically analyzed.

17-alpha-hydroxyprogesterone dose[1] (μg g^{-1})	First ejaculation[2]		Second ejaculation[3]	
	N	sperm count (millions)	N	sperm count (millions)
0.10	4	$0.16^{a} \pm 0.32$	4	$2.34^{a} \pm 2.70$
0.01	4	$2.84^{a} \pm 5.67$	4	$3.32^{a} \pm 4.83$
Control[4]	4	$0.46^{a} \pm 0.55$	4	$1.70^{a} \pm 1.23$

[1] Single injection per individual. Mean body weight = 22.2 ± 1.5 g.

[2] 14 d after hormone injection.

[3] 28 d after hormone injection. Survival rates were 100% for each treatment.

[4] Control = 0.1 μl ethanol g^{-1} body weight in 50 μl saline solution.

or better results for sperm count and maybe reduce the speed of the deterioration process, but this needs further investigation.

In contrast 17-alpha-hydroxyprogesterone did not induce a significant improvement in spermatophore quality under our experimental conditions. Sperm counts were very low in each treatment including the control; additionally, spermatophores were small and presented a high incidence of abnormalities. Progesterone has been effective on ovarian maturation in *Metapenaeus ensis* (Yano, 1985) and 17-alpha-hydroxyprogesterone has induced vitellogenin secretion in *P. japonicus* (Yano, 1987).

17-alpha-methyltestosterone molecule enhanced spermatophore quality. Additionally, Nagabhushanam and Kul Karni (1981) reported an enhancement effect of testosterone propionate and testosterone acetate on androgenic gland activity and spermatogenesis in *Parapenaeopsis hardwickii*. These results could be the consequence of a pharmacological effect, activating spermatogenesis/spermatophore synthesis mechanisms by high doses of non physiologically related molecules or it could be that these molecules are complementary to AGH for the intensification of spermatogenesis/spermatophore synthesis.

Regardless of these physiological implications, from an aquaculture point of view this finding indicates a practical way of improving spermatophore quality. Contrary to the physiological disadvantages (Quakenbush, 1986) of unilateral eyestalk ablation, a steroid injection program seems to be practical and manageable. However, further research is required for its implementation.

Acknowledgments

The author is grateful for the support of the aquaculture programs: UNA-LUW and UNA-JICA, Escuela de Ciencias Biológicas, and Criadero de Camarones de Chomes S.A. To Dr. Jorge Gunther, Dr. Karel Kleijn, M.Sc. Clemencia León, and journal reviewers for their comments on the manuscript.

References

Alfaro, J., 1990. A contribution to the understanding and control of the male reproductive system melanization disease of broodstock *Penaeus setiferus*. Master's Thesis, Department of Wildlife and Fisheries Sciences, Texas A & M University, USA.

Alfaro, J., 1993. Reproductive quality evaluation of male *Penaeus stylirostris* from a grow-out pond. J. World Aquacult. Soc. 24, 6 - 11.

Alfaro, J., 1994. Ultraestructura de la glándula androgénica, espermatogénesis y oogénesis de camarones marinos (Decapoda: Penaeidae). Rev. Biol. Trop. 42, 121 - 129.

Alfaro, J., Lozano, X., 1993. Production and deterioration of spermatophores in pond-reared *Penaeus vannamei*. J. World Aquacult. Soc. 24, 522 - 529.

Bray, W. A., Leung-Trujillo, J., Lawrence, A. L., 1985. Preliminary investigation on the effects of temperature, bacterial inoculation, and EDTA on sperm quality in captive *Penaeus setiferus*. J. World Maric. Soc. 16, 250 - 257.

Browdy, C., 1992. A review of the reproductive biology of *Penaeus* species: perspectives on controlled shrimp maturation systems for high quality nauplii production. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming. World Aquaculture Society, Baton Rouge, Louisiana, USA, pp. 22 – 51.

Chamberlain, G. W., 1988. Stepwise investigation of environmental and nutritional requirements for reproduction of penaeid shrimp. PhD Thesis, Department of Wildlife and Fisheries Sciences, Texas A&M University, USA.

Charniaux-Cotton, H., 1960. Sex determination. In: Waterman, T. H. (Ed.), The physiology of crustacea, vol. 1 Metabolism and growth. Academic Press, New York, New York, USA, pp. 411 - 447.

Charniaux-Cotton, H., Payen, G., 1988. Crustacean reproduction. In: Downer, R. G. H., and Laufer, H. (Eds), Invertebrate endocrinology, vol. 2. Alan. R. Liss, Inc., New York, New York, USA, pp. 279 – 303.

Charniaux-Cotton, H., Zerbib, C., Meusy, J. J., 1966. Monographie de la glande androgene des crustaces superieurs. Crustaceana (Leiden) 10, 113 - 136.

Dougherty, W. J., Dougherty, M. M., 1989. Electron microscopical and histochemical observations on melanized sperm and spermatophores of pond-cultured shrimp, *Penaeus vannamei*. J. Invertebr. Pathol. 54, 331 - 343.

Fingerman, M., 1987. The endocrine mechanisms of crustaceans. J. Crustacean Biol. 7, 1 - 24.

Griffin, F. J., Clark, W. H. Jr., Crowe, J. H., Crowe, L. M., 1987. Intracellular pH decreases during the *in vitro* induction of the acrosome reaction in the sperm of *Sicyonia ingentis*. Biol. Bull. 173, 311 - 323.

Hasegawa, Y., Haino-Fukushima, K., Katakura, Y., 1987. Isolation and properties of androgenic gland hormone from the terrestrial isopod, *Armadillidium vulgare*. Gen. Comp. Endocrin. 67, 101 - 110.

Heitzmann, J. C., Diter, A., AQUACOP, 1993. Spermatophore formation in the white shrimp, *Penaeus vannamei* Boone 1931: dependence on the intermoult cycle. Aquaculture 116, 91 - 98.

Huquet, D., Huquet, P., 1971. La glande androgene de *Palaemon serratus* (Pennant), *Crangon crangon* (Linné), *Aristeus antennatus* (Risso). B. Mus. Natl. Hist. Nat. 3(10), 597 - 610.

Leung-Trujillo, J. R., Lawrence, A. L., 1985. The effect of eyestalk ablation on spermatophore and sperm quality in *Penaeus vannamei*. J. World Maric. Soc. 16, 258 - 266.

Leung-Trujillo, J. R., Lawrence, A. L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65, 363 - 370.

Nagabhushanam, R., KulKarni, G. K., 1981. Effect of exogenous testosterone on the androgenic gland and testis of a marine prawn, *Parapenaeopsis hardwickii* (Miers) (Crustacea, Decapoda, Penaeidae). Aquaculture 23, 19 - 27.

Nagamine, C., Knight, A. W., Maggenti, A., Paxman, G., 1980a. Effects of androgenic gland ablation on male primary and sexual characteristics in the malaysian prawn, *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae), with first evidence of induced feminization in a nonhermaphroditic decapod. Gen. Comp. Endocrin. 41, 423 - 441.

Nagamine, C., Knight, A. W., Maggenti, A., Paxman, G., 1980b. Masculinization of female *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae) by androgenic gland implantation. Gen. Comp. Endocrin. 41, 442 - 457.

Ott, L., 1984. An introduction to statistical methods and data analysis. Second Ed., Duxbury Press, Boston, Massachusetts, USA, 775 pp.

Ottogalli, L., Galinie, C., Goxe, D., 1988. Reproduction in captivity of *Penaeus stylirostris* over ten generations in New Caledonia. J. Aquacult. Trop. 3, 111-125.

Payen, G. G., Chin, L., Laubier-Bonichon, A., Charniaux-Cotton, H., 1982. The androgenic gland of the shrimp *Penaeus japonicus* Bate. Description, role, and control by the eyestalk. Gen. Comp. Endocrin. 46 (3), 384.

Pratoomchat, B., Piyatiratitivorakul, S., Menasveta, P., 1993. Sperm quality of pond-reared and wild-caught *Penaeus monodon* in Thailand. J. World Maric. Soc. 24, 530 - 540.

Quackenbush, L. S., 1986. Crustacean endocrinology, a review. Can. J. Fish. Aquat. Sci. 43, 2271 - 2282.

Sagi, A., 1988. The androgenic gland in crustacea-with emphasis on the cultured freshwater prawn *Macrobrachium rosenbergii*- a review. Isr. J. Aquacult. (Bamidgeh) 40 (4), 107 - 112.

Taketomi, Y., Murata, M., Miyawaki, M., 1990. Androgenic gland and secondary sexual characters in the crayfish *Procambarus clarkii*. J. Crustacean Biol. 10, 492 - 497.

Talbot, P., Howard, D., Leung-Trujillo, J., Lee, T. W., Li, W. I., Ro, H., Lawrence, A. L., 1989. Characterization of male reproductive tract degenerative syndrome in captive penaeid shrimp (*Penaeus setiferus*). Aquaculture 78, 365 - 377.

Wang, Q., Misamore, M., Jiang, C. Q., Browdy, C. L., 1995. Egg water induced reaction and biostain assay of sperm from marine shrimp *Penaeus vannamei*: dietary effects on sperm quality. J. World Aquacult. Soc. 26, 261 - 271.

Yano, I., 1985. Induced ovarian maturation and spawning in greasyback shrimp, *Metapenaeus ensis*, by progesterone. Aquaculture 47, 223 - 229.

Yano, I., 1987. Effect of 17-alpha-hydroxy-progesterone on vitellogenin secretion in kuruma prawn, *Penaeus japonicus*. Aquaculture 61, 49 - 57.

Chapter 7

Ovarian Maturation and Spawning in the White Shrimp, *Penaeus vannamei*, by Serotonin Injection

A. Vaca, J. Alfaro

Escuela de Ciencias Biológicas, Universidad Nacional,
Heredia, Costa Rica

Published in: Aquaculture (2000), 182: 373 – 385

Abstract

The effect of serotonin (5-hydroxytriptamine, 5-HT) injection on the ovarian maturation and spawning of wild *Penaeus vannamei* was investigated. The neurotransmitter was evaluated at 15 μg g^{-1} body weight (b.w.) and 50 μg g^{-1} b.w., applied at day 1, 11 and 21. The effect was compared against a control group, which received the injection of the sterile vehicle solution (NaCl 0.85%), and an unilaterally eyestalk ablated group. 5-HT induced ovarian maturation and spawning at both doses tested, generating more spawnings at 50 μg g^{-1} b.w.; however, unilateral eyestalk ablation induced a sooner and a higher rate of maturation and spawning. Our findings may be the result of 5-HT interaction with the release of different neurohormones and inhibition of methyl farnesoate synthesis. Additionally, gonad inhibiting hormone may have an intense control over ovaries and hepatopancreas. Spawnings obtained by serotonin treatment showed excellent quality, and it was not statistically different ($P > 0.05$) from unilaterally eyestalk ablated females. Further research is necessary in order to increase the spawning activity and define the duration of the reproductive performance of serotonin treated females.

Key-words: *Penaeus vannamei*; Reproduction; Serotonin; Shrimp

Introduction

Panouse (1943) discovered that removal of both eyestalks from *Palaemon serratus* resulted in precocious ovarian development; 30 years later this endocrinological approach was applied to *Penaeus duorarum* (Caillouet, 1972) by unilateral eyestalk ablation, and subsequently confirmed in other shrimp species (Arnstein and Beard, 1975; Primavera, 1978; Aquacop, 1979).

Worldwide commercial maturation of female penaeids relies almost exclusively on the technique of unilateral eyestalk ablation (Browdy, 1992; Fingerman, 1997a); the technique gives predictable peaks of maturation and spawning, but many associated problems have been reported, like deterioration in spawn quality and quantity over time (Emmerson, 1980; Primavera, 1985; Tsukimura and Kamemoto, 1991), and conflicting results on spawn size, hatch succcess and other variables (Browdy, 1992).

Eyestalks are the endocrine center for regulating many physiological mechanisms, such as molting, metabolism, sugar balance, heart rate, pigments, and gonad maturation. Therefore, unilateral eyestalk ablation affects all aspects of shrimp physiology (Quackenbush, 1986). Predictable induced reproduction in captive penaeids without the use of eyestalk ablation was considered a long term goal for shrimp mariculture (Quackenbush, 1991).

Various alternatives to ablation have been evaluated, based on accumulated knowledge about environmental control and crustacean endocrinology. Photoperiod and temperature manipulations, based on seasonal natural variations of these parameters, have been successful in controlling maturation of unablated *P. japonicus* (Laubier-Bonichon, 1978), *P. stylirostris* (Chamberlain and Gervais, 1984), and *P. setiferus* (Chamberlain, 1988); however, photoperiod control seems to be more important for subtropical species (for review: Browdy, 1992). Implantation of thoracic ganglia from a mature female into an immature female stimulates vitellogenesis (Otsu, 1963). This principle was tested on *P. vannamei* by implanting thoracic ganglia from maturing *Homarus americanus* (Yano et al., 1988); the report indicates that the implantation technique generates maturation, but the experiment is based on a low number of replicates, without statistical analysis. Moreover, the current knowledge on tissue recognition between different crustacean species is limited, so there is no scientific evidence to support the assumption that tissue from a lobster would be recognized as self by a shrimp (Crompton, 1967; Lackie, 1986).

Steroids are biologically active in crustaceans and they have been found in ovarian tissue of *P. monodon* at different stages of vitellogenesis (Fairs et al., 1990). The authors proposed that ovarian development and oocyte maturation in crustaceans may be regulated by steroid hormones similar to teleost fish and amphibia. Complementary, it has been established that the mandibular organ of crustaceans synthesizes and secretes steroids and terpenoids, which may play a role in ovarian stimulation (Tsukimura and Kamemoto, 1991). This study found that 17 alpha-hydroxyprogesterone, methyl farnesoate and juvenile hormone III significantly increased *P. vannamei* oocyte diameter, *in vitro*.

Injection of progesterone was tested in *Metapenaeus ensis* (Yano, 1985); the study shows a possible induction of maturation without statistical confirmation, and quality of spawnings could not be evaluated. Complementary, 17 alpha-hydroxyprogesterone induced vitellogenin secretion in *P. japonicus* (Yano, 1987). Alfaro (1996) demonstrated a significant improvement in spermatophore quality by 17 alpha-methyltestosterone injection in *P. vannamei*.

A completely new approach to induce gonadal maturation and spawning was defined by Fingerman (1997b); this approach relies on the role that neurotransmitters play in regulating gonadal maturation. Sarojini et al. (1995) established that 5 - hydroxytryptamine (5-HT, serotonin) induces ovarian maturation *in vivo* and *in vitro*, in *Procambarus clarkii*, by acting to stimulate release of a gonad- stimulating hormone (GSH) from the brain and thoracic ganglia; GSH is an abstract entity, which has not been identified nor measured directly.

Our current knowledge on regulation of GSH and gonad - inhibiting hormone (GIH) release was presented by Fingerman (1997b). GSH release is stimulated by 5-HT and red pigment - concentrating hormone (RPCH); GSH release is inhibited by dopamine (DA) and methionine enkephalin (Met-ENK); GIH release is stimulated by Met-ENK and DA, and GIH release inhibition has not been reported.

Serotonin also stimulates the release of other neurohormones, including the crustacean hyperglycemic hormone (CHH), red pigment-dispersing hormone (RPDH), neurodepressing hormone (NDH) and molt-inhibiting hormone (MIH; Sarojini et al., 1994). Methyl farnesoate (MF) is a factor that stimulates gonadal maturation (Laufer et al., 1993) and its synthesis is inhibited by 5-HT; on the other hand, RPCH stimulates MF synthesis by the mandibular organ (Fingerman, 1997b). Other roles played by 5-HT are migration of the proximal retinal pigment, pericardial organ neurohormone, stomatogastric ganglion neuromodulator or neurohormone, behavioral responses, osmoregulation, and mechanoreception (Fingerman et al., 1994).

As an effort to develop alternatives for the commercial reproduction of penaeid shrimp, this study was conducted to evaluate the effect of two doses of 5-HT on *P. vannamei* maturation and spawning quality, compared to unilateral eyestalk ablation.

Material and methods

Broodstock

Adult *P. vannamei* with a mean weight of 56 g for females and 40 g for males, were obtained by trawling off Golfo de Panamá, on the Pacific coast. Animals were transported in oxygenated seawater to the Larvae Production Center of Coclé shrimp farm, Panamá. Selection was done based on size, stage of ovarian maturation, and healthy appearance.

Experimental conditions

Maturation system consisted of 3 concrete tanks (26 m^2 / each), with a water depth of 0.35 m and total daily replacement of 200%. Light intensity was approximately 2 $\mu Em^{-2}s^{-1}$, with a photoperiod of 8 h light: 16 h dark; these conditions are routinely used in the shrimp farm. Water temperature was 29.5 ± 0.8 °C, salinity between 30 and 34 ppt. Animals were fed at 21% body weight/ day with fresh frozen squids, bloodworms and oysters at a ratio of 1:1:1, respectively.

Molting and reproduction are major metabolic processes in decapod Crustacea (Adiyodi and Adiyodi, 1970); therefore, in penaeid shrimp ovarian maturation and spawning occur during the intermolt (Browdy, 1992). The molt cycle duration of adult *Penaeus* is 11 – 14 days (Robertson et al., 1987); at postmolt females are weak and at premolt maturation is delayed. During the adaptation period (2 weeks) to the

experimental environment, molting stages of females were determined by uropod analysis, based on the technique described by Robertson et al. (1987). Intermolt females (2 – 3 days) were randomly distributed in a block design, integrated by 3 tanks with 4 treatments each, and 12 females per treatment-tank, for a total number of 36 females per treatment. Each female was individually eyestalk tagged; tank density was adjusted to 5 animals m^{-2} using non-treated animals, with 1:1 sex ratio.

Treatment A consisted of unilateral eyestalk ablation by cutting the right eyestalk with red-heated scissors; animals in treatment B received 3 injections (days 1, 11 and 21) of 5-HT creatinine sulfate (Sigma Chemical Co., St. Louis, Missouri, USA) at 15 μg g^{-1} body weight (b.w., Sarojini et al., 1995). Treatment C received 3 injections (days 1, 11 and 21) of 5-HT at 50 μg g^{-1} b.w. Treatment D served as control receiving 3 injections of sterile vehicle solution (NaCl 0.85%), at the same experimental days. Volume of injection was 0.07 ml per 56 g; the study was undertaken for 46 days.

Ovarian maturation was evaluated by external observation of ovarian size and color as described by King (1948) and Yano et al. (1988) with slight modifications:

Stage I. The ovary is transparent with no distinguishable outline.

Stage II. The ovary is visible as a thin opaque line along the dorsal central axis.

Stage III. The ovary is visible as a thick and yellow band.

Stage IV. The ovary is turgid, broad and dark orange. Mating and spawning are imminent.

Quality of spawns was evaluated in terms of number of eggs per spawn, percentage of fertilized eggs, hatching rate, and number of nauplii per spawn. Mature and mated females were selected and placed in individual spawning tanks (80 l); 10 h after spawning, the eggs were washed and concentrated in a 10-l container, three 1-ml samples were taken to asses total number of eggs, additional samples were taken for a microscopic investigation of the proportion of normally embryonal eggs vs. non-embryonal eggs (Primavera and Posadas, 1981). Eggs were treated with formalin (37%) and iodine (1%) at 0.26 ml l^{-1} and 0.20 ml l^{-1}, respectively; then moved to hatching containers (60 l) at salinity of 30 ppt. Thirty-four hours after spawning, nauplii were collected and concentrated in 15 l containers; three samples of 1 ml were randomly taken to count number of nauplii. Hatching rate was estimated based on number of eggs and nauplii produced per spawn.

Statistical analysis

Experimental data were analyzed with Statgraphics plus (version 7.0), for one way classification analysis of variance (AOV) and Duncan´s multiple range procedure. Prior to analysis, data on percentages were transformed using squared root of arcsine

(Bray et al., 1990), to make the variance independent of the mean; alpha levels for all tests were set at 0.05. Untransformed data are presented as mean ± standard error (s.e.).

Results

Mortality during experimental period was similar between treatments, no statistical differences were measured ($P > 0.05$); however, eyestalk ablation gave the highest variance between tanks (Table 1).

P. vannamei was induced to mature by eyestalk ablation as well as by 5-HT injection (Fig. 1). Following unilateral eyestalk ablation, animals started to develop ovaries; the weekly analysis shows a high rate of maturation through day 7, 14, 21 and 35 for ablated shrimp; treatments with 50 μg 5-HT and 15 μg 5-HT induced also maturation, starting on day 14 and generating a low rate of induction during the experimental period.

Table 1 shows the rate at which females were induced to mature and spawn successfully under the protocols tested. All the eyestalk ablated females reached the spawning condition, whereas the injection of 15 μg g^{-1} and 50 μg g^{-1} b.w. of 5-HT induced spawning of 20% and 35% of the females, respectively. As it can be seen in Table 1, ablation allowed a great proportion of females to re-mature and spawn many times, and 5-HT only induced re-maturation and spawning in few animals (6% - 7%) during the 46 days of experimentation.

Fig. 2 shows the daily spawning activity for each treatment; ablation is by far the best technique tested, although 5-HT treatments also generated consecutive spawnings. Table 2 presents a statistical comparison in spawning activity for weekly intervals; the analysis indicates that the spawning activity for ablated females increased to a maximum performance of 95% of females at week 4. The spawning activity for serotonin treated females was maximum at week 4 and 6 for both doses. Complementary, in the control group only 2 spawns occurred, representing 6.7 % of the group.

Quality of spawnings is presented in Table 3; the variation of the number of eggs obtained were not statistically different between treatments for first spawnings nor between first, second, third, fourth and fifth spawnings for the ablated animals ($P > 0.05$). Second spawnings for the other treatments were not statistically analyzed because of the low number of replicates. A similar trend was observed for the percentage of fertilized eggs, percentage of hatch and number of nauplii per spawn.

Table 1. Rate of females reaching spawning during the experimental period for *Penaeus vannamei* treated with different protocols. b.w.: body weight.

Variable	Treatment group			
	Ablation (Mean ± s.e)	50 µg/g b.w. (Mean ± s.e)	15 µg/g b.w. (Mean ± s.e)	Saline solution (NaCl 0.85%) (Mean ± s.e)
Females in first spawning (%)[a]	100a	35.4 ± 1.8b	19.5 ± 14.6b	6.1 ± 10.5c
Females in second spawning (%)	78.0 ± 9.8	6.1 ± 5.3	6.7 ± 5.9	0
Females in third spawning (%)	40.9 ± 19.4	0	0	0
Females in fourth spawning (%)	23.7 ± 22.8	0	0	0
Females in fifth spawning (%)	7.0 ± 6.1	0	0	0
Mortality (%)[a]	44.4 ± 31.5a	36.1 ± 19.2a	33.3 ± 8.3a	33.3 ± 8 .3a

[a] Means with different letters indicate statistically significant differences ($P<0.05$).

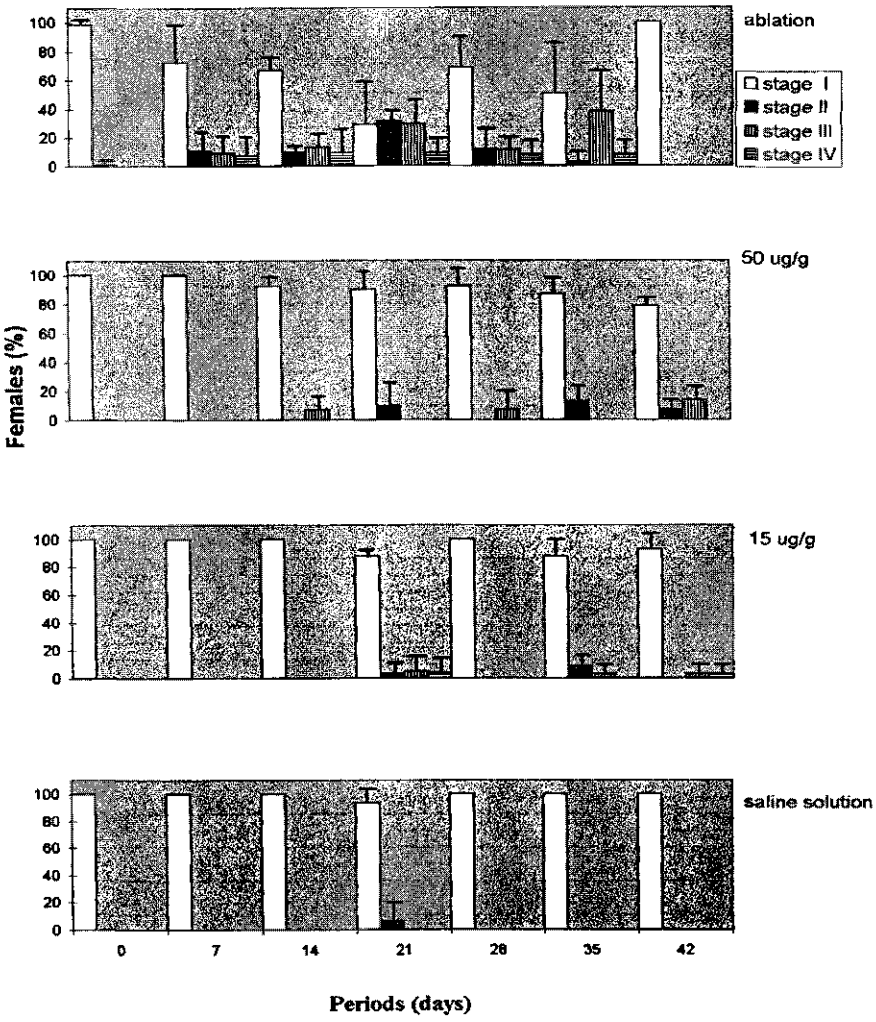

Figure 1. Stages of ovarian maturation (mean ± s.e.), registered every 7 days, for *P. vannamei* treated with different protocols.

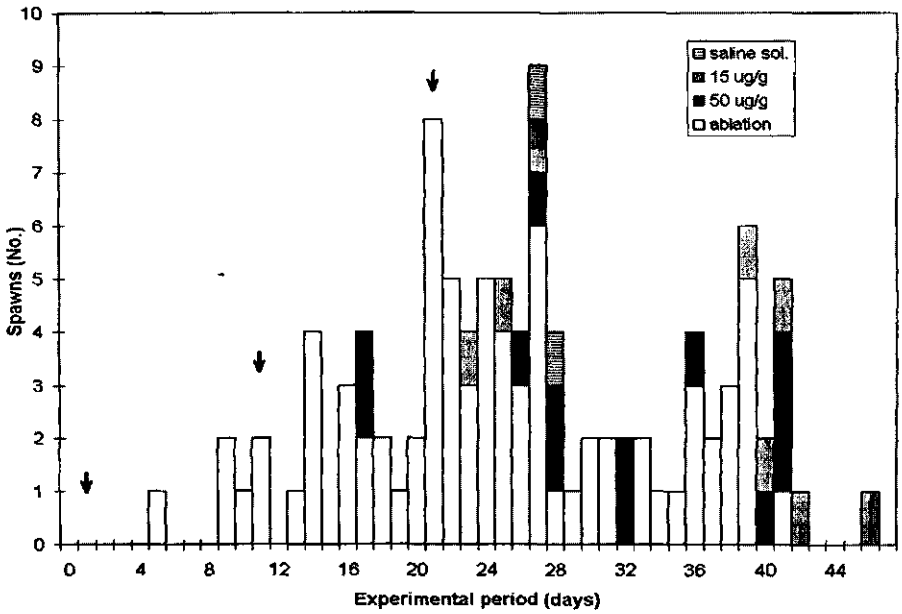

Figure 2. Daily spawning activity of *P. vannamei* treated by different protocols. ↓ = days when injections were applied.

Discussion

To our knowledge this is the first report that demonstrates maturation and spawning by 5-HT injection in a penaeid shrimp. Based on the findings by Fingerman (1997b), 5-HT activated the release of GSH, producing gonad maturation and spawning in *P. vannamei.*

Ablated females started to mature 3 days after ablation and they kept high rates of maturation until day 41 (week 6); during week 7 no maturation was observed in ablated females. Similar patterns have been previously reported, for example, Chamberlain and Gervais (1984) obtained spawning activity during 45 days for ablated *P. stylirostris*, and Simon (1982) reported spawning activity during 6 and 8 weeks for ablated *P. monodon*. This seems to indicate that the intense spawning activity induced by ablation generates a physiological unbalance, possibly involving nutrient depletion from the hepatopancreas (Lumare, 1979). This disorder deactivates vitellogenesis, stopping the ovarian maturation process until the female re-establish an acceptable physiological balance. On the other hand, serotonin treatment gave spawning activity at week 7, which could indicate that more spawnings would be expected in the following

Table 2. Weekly spawning rate of *Penaeus vannamei* treated by different protocols. Means with different letters within row indicate statistically significant different protocols ($P < 0.05$). b.w.: body weight.

Treatment group	Females (%)						
	Week 1[a] (mean ± s.e.)	Week 2[a] (mean ± s.e.)	Week 3 (mean ± s.e.)	Week 4[a] (mean ± s.e.)	Week 5 (mean ± s.e.)	Week 6 (mean ± s.e.)	Week 7 (mean ± s.e.)
Ablation	3.7 ± 6.4a	33.0 ± 22.8b	58.0 ± 2.2b	95.2 ± 8.3c	39.9 ± 25.2b	62.3 ± 21.7b	0a
n	31	31	31	28	23	22	20
5-HT 50µg/g b.w.	0a	0a	7.5 ± 6.6bc	14.8 ± 5.0cd	3.7 ± 6.4 ab	20.4 ± 11.5d	0a
n	31	30	28	27	24	24	23
5-HT 15 µg/g b.w.	0a	0a	0a	12.3 ± 2.2c	0a	15.9± 16.7bc	3.7± 6.4ab
n	29	25	25	25	24	24	24
Saline solution (NaCl 0.85%)	0a	0a	0a	6.7 ± 11.5a	0a	0a	0a
n	31	29	28	26	24	24	24

[a] Weeks when injections were applied.

Table 3. Reproductive performance variables of *Penaeus vannamei* females treated with different protocols. Means for each variable where not statistically different ($P > 0.05$) between treatments for first spawning, and between spawnings for ablated females. b.w.: body weight.

Variable	Treatment group							
	Ablation		5-HT 50µg/g b.w.		5-HT 15µg/g b.w.		Saline solution (NaCl 0.85%)	
	n	Mean ± s.e.	n	Mean ± s.e.	n	Mean ± s.e.	n	Mean ± s.e.
Eggs/ spawn								
First spawning	31	194446 ± 63737	11	206666 ± 55597	6	218333 ± 77079	2	228333 ± 120208
Second spawning	24	217021 ± 61475	2	166667 ± 4714	2	186667 ± 23570		-
Third spawning	13	182026 ± 61440		-		-		-
Fourth spawning	8	217083 ± 41536		-		-		-
Fifth spawning	2	161663 ± 7070		-		-		-
Fertilized eggs (%)								
First spawning		70.3 ± 31.1		68.0 ± 27.5		71.8 ± 21.6		49.5 ± 17.3
Second spawning		59.9 ± 26.3		84.1 ± 8.06		69.6 ± 29.1		-
Third spawning		66.8 ± 30.8		-		-		-
Fourth spawning		45.7 ± 34.9		-		-		-
Fifth spawning		45.5 ± 40.3		-		-		-

Hatching rate (%)				
First spawning	52.1 ± 27.2	58.2 ± 28.1	57.1 ± 8.7	62.4 ± 4.3
Second spawning	49.7 ± 24.6	58.9 ± 29.3	21.1 ± 4.9	-
Third spawning	59.2 ± 30.4	-	-	-
Fourth spawning	38.1 ± 37.0	-	-	-
Fifth spawning	58.1 ± 32.5	-	-	-
Nauplii/ spawn (%)				
First spawning	100389 ± 57018	109364 ± 49694	113000 ± 28531	145000 ± 34853
Second spawning	106458 ± 62424	97500 ± 45962	40000 ± 14142	-
Third spawning	117692 ± 75791	-	-	-
Fourth spawning	81250 ± 78000	-	-	-
Fifth spawning	95000 ± 56569	-	-	-

weeks; however, research has to be implemented to define if serotonin can generate a longer reproductive performance than eyestalk ablation.

Removal of one eyestalk induced a sooner and higher rate of maturation and spawning compared to 5-HT treatment. This observation may indicate that GIH control over ovaries and hepatopancreas (Quackenbush, 1991) is very intense; however, other factors should be considered. Serotonin induces hyperglycemia in decapods by releasing the crustacean hyperglycemic hormone (CHH; Keller and Beyer, 1968); on the other hand, eyestalk ablation induces hypoglycemia (Keller, 1974). Since it has not been established any direct effect of CHH on decapod maturation (Keller et al., 1985; Fingerman, 1997a), the elevated glucose level measured in the hemolymph of *P. vannamei* after 5-HT injection may have a negative effect on maturation (Racotta and Palacios, 1998) since 5-HT could also play a role as a stress modulator (Lüschen et al.,

1993). Additionally, the inhibitory effect of 5-HT on MF synthesis may have contributed to the lower rate of maturation and spawning in the 5-HT treatment.

5-HT stimulation on the release of MIH (Mattson and Spaziani, 1985) may have a positive effect on maturation because MIH inhibits the secretion of ecdysone by the Y– organ, delaying the molting event (Chang, 1985). However, ecdysteroids seem to play a role in ovulation and embryonic development in Crustacea (Wilder and Aida, 1995), so during ovarian maturation the molting hormone (20-hydroxyecdysone) secretion is inhibited, but other ecdysteroids are being accumulated in developing oocytes (Wilder and Aida, 1995).

The data on spawning quality presented in this report, demonstrate that once the gonad maturation mechanism is activated by 5-HT, the animal will generate eggs and nauplii at commercial numbers, similar to previous reports for eyestalk ablated *P. vannamei*. Regardless of treatment and re-maturation, 56 g females generated an average of 200,000 eggs per spawn, with a 50% hatching rate; these values agree with the commercial production of 55,000 to 150,000 nauplii per spawn for ablated *P. vannamei* (Kawahigashi, 1992) and the number of eggs per spawn reported by Wyban et al. (1992) and Lotz and Ogle (1994) of 119,000 and 152,432, respectively.

Based on our experimental data, a serotonin injection program seems to be a practical alternative to eyestalk ablation; however, further research is required to increase the spawning activity and evaluate the duration of the reproductive performance for serotonin treated females. The release of different neurohormones (GSH, MIH, CHH, RPDH, and NHH) may be controlled by a difference in the threshold of 5-HT; therefore, it would be interesting to screen the effect of different 5-HT concentrations on shrimp maturation.

The doses used in this study seem to be pharmacological; 15 μg g^{-1} b.w. was based on Sarojini et al. (1995) for *Procambarus clarkii*. To our knowledge the physiological level of 5-HT in *P. vannamei* hemolymph has not been reported; in the crab, *Libinia emarginata*, 10^{-8} M 5-HT inhibited MF synthesis by 20-35%, 10^{-6} M 5-HT enhanced the contraction of foregut neuromuscular preparations of *Panulirus interruptus* and *Cancer magister*, a high dose of 5-HT (10^{-4} M) caused extreme leg flexion in *Carcinus maenas* (for review: Fingerman et al., 1994). An injection of 15 μg and 50 μg would give an estimated hemolymph level in the order of 10^{-5} and 10^{-4} M, respectively, considering the relation of 0.5 ml hemolymph per gram of shrimp (Shafir et al., 1992).

Acknowledgements

The authors appreciate the interest and support of the company Coclé Shrimp Farm, from Panamá. Special thanks to its general manager, Ing. Adriano González,

whose cooperation was crucial for the implementation of this research. To Geoffrey Gearheart for manuscript review, and to unknown referees for their valuable comments.

References

Adiyodi, K. G., Adiyodi, R. G., 1970. Endocrine control of reproduction in decapod crustacea. Biol. Rev. 45, 121 - 165.

Alfaro, J., 1996. Effect of 17 alpha-methyltestosterone and 17 alpha-hydroxyprogesterone on the quality of white shrimp *Penaeus vannamei* spermatophores. J. World Aquacult. Soc. 27(4), 487 - 492.

Aquacop, 1979. Penaeid reared broodstock: closing the cycle of *P. monodon*, *P. stylirostris* and *P. vannamei*. Proc. World Maric. Soc. 10, 445 - 452.

Arnstein, D.R., Beard, T.W., 1975. Induced maturation of the prawn *Penaeus orientalis* Kishinouye in the laboratory by means of eyestalk removal. Aquaculture 5, 411 - 412.

Bray, W.A., Lawrence, A.L., Lester, L.J., 1990. Reproduction of eyestalk-ablated *Penaeus stylirostris* fed various levels of total dietary lipid. J. World Aquacult. Soc. 21(1), 41 - 52.

Browdy, C.L., 1992. A review of the reproductive biology of penaeus species: Perspectives on controlled shrimp maturation systems for high quality nauplii production. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 22-51.

Caillouet, A.C.,Jr., 1972. Ovarian maturation induced by eyestalk ablation in pink shrimp, *Penaeus duorarum* Burkenroad. Proc. World Maric. Soc. 3, 205 - 225.

Chamberlain, G. W., 1988. Stepwise investigation of environmental and nutritional requirements for reproduction of penaeid shrimp. PhD Thesis, Department of Wildlife and Fisheries Sciences, Texas A&M University, USA.

Chamberlain, G.W., Gervais, N.F., 1984. Comparison of unilateral eyestalk ablation with environmental control for ovarian maturation of *Penaeus stylirostris*. J. World Maric. Soc. 15, 29 - 30.

Chang, E. S., 1985. Hormonal control of molting in decapod crustacea. Am. Zool. 25, 179 - 185.

Crompton, D. W., 1967. Studies on the haemocytic reaction of *Gammarus* sp., and its relationship to *Polymorphus minutus* (Acanthocephala). Parasitology 57, 389 - 401.

Emmerson, W.D., 1980. Induced maturation of prawn *Penaeus indicus*. Mar. Ecol. Prog. Ser. 2, 121 - 131.

Fairs, N. J., Quinlan, P. T., Goad, L. J., 1990. Changes in ovarian unconjugated and conjugated steroid titers during vitellogenesis in *Penaeus monodon*. Aquaculture 89, 83 - 99.

Fingerman, M., Nagabhushanam, R., Sarojini, R., Reddy, P. S., 1994. Biogenic amines in crustaceans: identification, localization, and roles. J. Crustacean Biol. 14 (3), 413 - 437.

Fingerman, M., 1997a. Crustacean endocrinology: a retrospective, prospective, and introspective analysis. Physiol. Zool. 70(3), 257 - 269.

Fingerman, M., 1997b. Roles of neurotransmitters in regulating reproductive hormone release and gonadal maturation in decapod crustaceans. Invertebr. Reprod. Dev. 31, 47 - 54.

Kawahigashi, D., 1992. A survey of commercial maturation technology in the western hemisphere. In: Wyban, J. (Ed.), Proc. of the Special Sesion on Shrimp Farming, World Aquaculture Society, Baton Rouge, L.A., USA, pp. 52-54.

Keller, R., 1974. Stoffwechselregulation durch Neurohormone bei Crustaceen. Fortschr. Zool. 22, 34 - 54.

Keller, R., Beyer, J., 1968. Zur hyperglykämischen Wirkung von Serotonin und Augenstielextrakt beim Flusskrebs *Orconectes limosus*. Z. Vgl. Physiol. 59, 78 - 85.

Keller, R., Jaros, P., Kegel, G., 1985. Crustacean hyperglycemic neuropeptides. Am. Zool. 25, 207 - 221.

King, J. E., 1948. A study of the reproductive organs of the common marine shrimp, *Penaeus setiferus* (Linnaeus). Biol. Bull. 94, 244 - 262.

Lackie, A. M., 1986. Transplantation immunity in arthropods: is immunorecognition merely wound-healing? In: Brehelin, M. (Ed.), Immunity in invertebrates, Spring-Verlag, New York, USA, pp. 125-138.

Laubier-Bonichon, A., 1978. Ecophysiologie de la reproduction chez la crevette *Penaeus japonicus* Trois annees d'experience en milieu controle. Oceanologica Acta 1 (2), 135 - 150.

Laufer, H., Ahl, J. S. B., Sagi, A., 1993. The role of juvenile hormones in crustacean reproduction. Am. Zool. 33, 365 - 374.

Lotz, M.J., Ogle, J.T., 1994. Reproductive performance of the white-legged shrimp *Penaeus vannamei* in recirculating seawater systems. J. World Aquacult. Soc. 25(3), 477 - 482.

Lumare, F., 1979. Reproduction of *Penaeus kerathurus* using eyestalk ablation. Aquaculture 18, 203 - 214.

Lüschen, W., Willig, A., Jaros, P. P., 1993. The role of biogenic amines in the control of blood glucose levels in the decapod crustacean, *Carcinus maenas* L. Comp. Biochem. Physiol. 105C, 291 - 296.

Mattson, M. P., Spaziani, E., 1985. 5-Hydroxytryptamine mediates release of molt-inhibiting hormone activity from isolated crab eyestalk ganglia. Biol. Bull. 169, 246 - 255.

Otsu, T., 1963. Bihormonal control of sexual cycle in the freshwater crab, *Potamon dehaani*. Embryologica 8, 1 - 20.

Panouse, J.B., 1943. Influence de l'ablation du pédoncule oculaire sur la croissance de l'ovaire chez la crevette *Leander serratus*. C. R. Acad. Sci. Paris 217, 553 - 555.

Primavera, H.J., 1978. Induced maturation and spawning in five-month-old *Penaeus monodon* Fabricius by eyestalk ablation. Aquaculture 13, 355 - 359.

Primavera, H.J., Posadas, R., 1981. Studies on the eggs quality of *Penaeus monodon* Fabricus, based on morphology and hatching rates. Aquaculture 22, 269 - 277.

Primavera, H.J., 1985. A review of maturation and reproduction in closed thelycum penaeids. In: Taki, Y., Primavera, J. H., and Llobrera, J. A. (Eds.), Proc. 1st Int. Conf. on the Culture of Penaeid Prawns/Shrimp, Aquaculture Department, SEAFDEC, Iloilo, Philippines, pp. 47 - 64.

Quackenbush, L.S., 1986. Crustacean endocrinology; a review. Can. J. Fish. Aquat. Sci. 43, 2271 - 2282.

Quackenbush, L. S., 1991. Regulation of vitellogenesis in penaeid shrimp. In: DeLoach, P., Dougherty, W. J. and Davidson, M. A. (Eds.), Frontiers in Shrimp Research, Elsevier, Amsterdam, pp.125 - 140.

Racotta, I. S., Palacios, E., 1998. Hemolymph metabolic variables in response to experimental manipulation stress and serotonin injection in *Penaeus vannamei*. J. World Aquacult. Soc. 29 (3), 351 - 356.

Robertson, L., Bray, W., Leung-Trujillo, J., Lawrence, A., 1987. Practical molt staging of *Penaeus setiferus* and *Penaeus stylirostris*. J. World Aquacult. Soc. 18 (3), 180 - 185.

Sarojini, R., Nagabhushanam, R., Fingerman, M., 1994. 5-hydroxytryptaminergic control of testes development through the androgenic gland in the red swamp crayfish, *Procambarus clarkii*. Invertebr. Reprod. Dev. 26 (2), 127 - 132.

Sarojini, R., Nagabhushanam, R., Fingerman, M., 1995. Mode of action of the neurotransmitter 5-hydroxytryptamine in stimulating ovarian maturation in the red swamp crayfish, *Procambarus clarkii*: An *in vivo* and *in vitro* study. J. Exp. Zool. 271, 395 - 400.

Shafir, S., Tom, M., Ovadia, M., Lubzens, E., 1992. Protein, vitellogenin, and vitellin levels in the hemolymph and ovaries during ovarian development in *Penaeus semisulcatus* (de Haan). Biol. Bull. 183, 394 - 400.

Simon, C. M., 1982. Large-scale commercial application of penaeid shrimp maturation technology. J. World Maric. Soc. 13, 301 - 312.

Tsukimura, B., Kamemoto, F.I., 1991. *In vitro* stimulation of oocytes by presumptive mandibular organ secretions in the shrimp, *Penaeus vannamei*. Aquaculture 92, 59 - 66.

Wilder, M. N., Aida, K., 1995. Crustacean ecdysteroids and juvenoids: chemistry and physiological roles in two species of prawn, *Macrobrachium rosenbergii* and *Penaeus japonicus*. Isr. J. Aquacult. (Bamidgeh) 47, 129 - 136.

Wyban, J.A., Swingle, J.S., Sweeny, J.N., Pruder, G.D., 1992. Development of high health shrimp using specific pathogen free (spf) broodstock *Penaeus vannamei*. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 254-260.

Yano, I., 1985. Induced ovarian maturation and spawning in greasyback shrimp, *Metapenaeus ensis*, by progesterone. Aquaculture 47, 223 - 229.

Yano, I., 1987. Effect of 17α- hydroxy-progesterone on vitellogenin secretion in kuruma prawn, *Penaeus japonicus*. Aquaculture 61, 49 - 57.

Yano, I., Tsukimura, B.T., Sweeney, J.N., Wyban, J.A., 1988. Induced ovarian maturation of *Penaeus vannamei* by implantation of lobster ganglion. J. World Aquacult. Soc. 19 (4), 204 - 209.

Chapter 8

Cooling, Cryoprotectant and Hypersaline Sensitivity of Penaeid Shrimp Embryos and Nauplius Larvae

Jorge Alfaro [a], J. Komen [b], E.A. Huisman [b]

[a] Estación de Biología Marina, Escuela de Ciencias Biológicas, Universidad Nacional, Puntarenas, Costa Rica

[b] Fish Culture and Fisheries Group, Department of Animal Sciences, Wageningen Agricultural University, Netherlands

Published in: Aquaculture (2001), 195: 353 - 366

Abstract

The sensitivity of embryos of the penaeid shrimp, *Trachypenaeus byrdi*, to cooling, cryoprotectant exposure (dimethyl sulfoxide: DMSO, sucrose, methanol and glycerol), and hypersaline treatment was assessed in order to gain basic knowledge for cryopreservation procedures. In addition, cooling and DMSO exposure was evaluated in *Penaeus stylirostris* and *T. byrdi* nauplii. Morulae and advanced embryos (setae development stage) showed tolerance to cooling at 10 °C, but were very sensitive to 0 °C exposure. Methanol exposure at 12 °C up to 2 M, was non toxic for advanced embryos. DMSO toxicity was intermediate; no statistical decrease in survival ($P > 0.05$) was measured at 0.5 M. Sucrose and glycerol were toxic to both embryo stages over 0.25 M and 0.5 M, respectively. Morulae were more resistant to hypersaline treatment at 55 ppt than advanced embryos. Nauplii showed a better tolerance to cooling and DMSO exposure than embryos. These findings are being applied to develop a cryogenic protocol for penaeid embryos.

Key-words: Cooling tolerance, Cryoprotectants, Embryos, Penaeids, Shrimp

Introduction

International trade for penaeid shrimp seedstock is based entirely on nauplii and postlarvae shipping, which requires packing in large amounts of seawater to ensure product viability. The concept of chilled transport has been applied to penaeid shrimp since a long time ago. In Central and South America nauplii sales have become a new commercial activity (Wilkenfeld, 1992); transport is in chilled and oxygenated water at 25000 nauplii l^{-1} (Kungvankij et al., 1986). *Penaeus vannamei* postlarvae (0.06 – 0.08 g) at a density of 30 – 40 g l^{-1} showed excellent survival after 6 h at 19 – 23 °C (Samocha and Lawrence, 1992); based on Clifford (1992), late postlarvae can withstand temperatures as low as 16 °C, and frequently packing is done at densities of 2000 – 3000 small postlarvae l^{-1}, chilled to 18 – 20 °C for over 24 h.

Cryopreservation of penaeid shrimp seedstock would make international trading more economical and efficient, and it has been suggested as an important advance for future breeding programs (Anchordoguy et al., 1988; Benzie, 1998). Sperm from the palaemonid shrimp, *Macrobrachium rosenbergii*, were cryopreserved with 10% glycerol (Chow et al., 1985), and sperm from the penaeid shrimp, *Sicyonia ingentis*, were cryopreserved at –196 °C , following freezing at –1 °C min^{-1} to –30 °C in the presence of 5% dimethyl sulfoxide (DMSO, Anchordoguy et al., 1988).

However, cryogenic techniques for penaeid eggs, embryos, or larvae have not yet been developed (Benzie, 1998; Subramoniam and Arun, 1999). *Penaeus* nauplii and protozoea tolerated freezing to –30 °C, but not to –196 °C (Baust and Lawrence, 1977).

More recently, it was shown that *P. indicus* nauplius IV tolerated freezing to –40 °C by slow freezing; *P. monodon* nauplii and protozoea I could be frozen to –70 °C (Subramoniam and Arun, 1999, for review).

Other invertebrate embryos or larvae, which have been successfully cryopreserved are sea urchin embryos (Asahima and Takahashi, 1979), mussel embryos (*Choromytilus chorus*; Gallardo et al., 1988), D larvae of the bivalves, *Tapes philippinarum* and *Crassostrea gigas* (McFadzen and Utting, 1990), embryos of the rotifer, *Brachionus plicatilis*, nauplii of *Artemia* (Alfaro, 1999) and larvae of the ragworm, *Nereis virens* (Subramoniam and Arun, 1999, for review). Khin-Maung et al. (1998) reported on the cryopreservation of the nauplius larvae of the barnacle, *Balanus amphitrite*.

Although penaeid embryos have received little attention for cryopreservation, some toxicological aspects have been studied in *P. indicus* embryos (Subramoniam and Arun, 1999, for review). In order to develop cryogenic protocols, embryos must be treated with cryoprotectants at non-toxic concentrations before freezing; during freezing embryos will dehydrate due to increased salt concentrations, but dehydration before freezing may improve survival (Robertson et al., 1988; Nowshari and Brem, 1998).

In a previous study we investigated cryopreservation of *Artemia* embryos and nauplii (Alfaro, 1999). Decapsulated embryos of *Artemia* were cryopreserved with 20% DMSO by capsule formation on dry ice (-79 °C) and over a metallic surface exposed to liquid nitrogen (–196 °C). Survival was between 17 and 19% (cooled control = 4 ± 3 %). Moreover, this survival was improved to 39 ± 10% by embryo dehydration in a hypersaline solution (330 g NaCl l^{-1}), followed by capsule formation at –196 °C. The lower temperature tolerated by *Artemia* instar I nauplii was only –33 °C, with 10% DMSO; survival was 46% after thawing at room temperature Although *Artemia* is unique among animals in the way embryos have adapted to tolerate dryness and freezing through glycerol protection (Green, 1965; Clegg et al., 1999), *Artemia* embryos were cryopreserved easily while nauplii were not.

In the present paper we investigate the capabilities of *Trachypenaeus byrdi* embryos to tolerate cooling, cryoprotectant exposure (DMSO, sucrose, methanol and glycerol), and hypersaline dehydration. In addition, some preliminary results on the sensitivity to low temperature and DMSO exposure of *P. stylirostris* and *T. byrdi* nauplii are presented.

T. byrdi and *P. stylirostris* are commercially important penaeid species in Costa Rica; we use *T. byrdi* embryos as a model for penaeid shrimp, because impregnated and mature females are found in Gulf of Nicoya all around the year; *P. stylirostris* is

more difficult to find at any time. The results present a first step towards the development of a cryopreservation protocol for penaeid shrimp embryos.

Material and methods

Animals

Embryos and nauplius larvae were obtained by spawning wild and naturally impregnated *T. byrdi* and wild *P. stylirostris* matured in captivity. *T. byrdi* were obtained by trawling off Golfo de Nicoya, on the Pacific coast of Costa Rica; females with turgid, broad and green ovaries were selected and transported in containers with constant aeration to the Estación de Biología Marina, Puntarenas. Adult *P. stylirostris* were captured with gill nets by local fishermen in Golfo de Nicoya, and transported to the experimental facility.

Fertilized spawnings from *T. byrdi* were obtained the first night following the capture. Fertilized spawnings from *P. stylirostris* were obtained after females were unilaterally eyestalk ablated, and naturally inseminated in a circular maturation tank (18 m^2). During the spawning period, animals were fed at 20% body weight / day with a mixture (1:1:1) of frozen squids, bloodworms and sardine.

Experimental

Cooling, cryoprotectant, and salinity experiments were performed using concentrated samples of morula embryos collected 2 h after spawning (Treece and Yates, 1988) and advanced embryos (setae development stage) collected 10 h after spawning; each experiment followed a completely randomized design, using the spawn of one female to minimize variability between experimental units (Ott, 1984). Females of both species were individually isolated in 150 l spawning tanks and embryos were siphoned from the bottom of the tank with a 5-mm diameter glass tube and concentrated in 50 ml of the same water used in spawning tanks: aged and 1-µm filtered natural seawater at 31 - 35 ppt (NSW). Embryo density was adjusted to 70 – 100 embryos ml^{-1}. Based on phototactic response (Browdy, 1992), high-quality nauplii (stages III and IV) were obtained by attracting them to a light source at the surface of the spawning tank; the density was adjusted to 40 – 60 nauplii ml^{-1} or at high density (280 – 485 nauplii ml^{-1}).

Cooling tolerance experiments

T. byrdi embryos were first cooled in one step transferring them from ambient temperature (27 °C) to seawater precooled at 10 °C and 0 °C, and kept in a refrigerator or ice bath at the specified temperature for 240 min; 1.0-ml samples were taken at 0, 30, 60, 120, 180 and 240 min of exposure and transferred to beakers with 20 ml of

cooled (10 °C) NSW; embryos were then incubated in a chamber at 27 – 28 °C until nauplius development. Each temperature was tested in a separate experiment.

Nauplii concentrates of *T. byrdi* at high density (280 – 485 nauplii ml^{-1}) were cooled in one step from ambient temperature to 1 °C and -1°C for 120 min (in separate experiments); 0.1-ml samples were taken at 30, 60 and 120 min, transferred to 20 ml of cooled (5-6 °C) NSW, and incubated until reaching ambient temperature.

Toxicity experiments

DMSO, glycerol (0, 0.5, 1.0, 2.0 and 5 M), sucrose and methanol (0, 0.25, 0.5, 1.0 and 2.0 M) solutions were prepared in 10 ml of NSW and precooled to 12 °C; 1 ml embryo concentrate was added to 10 ml assay solution and kept at 12 °C for 20 min. The solution was then diluted to 150 ml with cooled (12 °C) NSW and after 10 min was gently decanted to 10 ml, keeping the embryos at the bottom of the beaker. A second, identical, dilution step was performed, keeping a 20-ml embryo suspension which was transferred to an incubation chamber at 27 – 28 °C until nauplius development. The cryoprotectant dilution protocol adopted for these assays allowed 99.5% chemical removal. In total, four different experiments were performed, each with a different cryoprotectant.

Two additional experiments with nauplii were performed. Nauplii concentrates of *P. stylirostris* (40 – 60 nauplii ml^{-1}) were exposed to DMSO by mixing 1-ml nauplii concentrate with 10 ml of DMSO solutions (0 and 1.4 M) which were kept at 27 °C or cooled to 5 °C before; the exposure period was 20 min. *T. byrdi* nauplii were similarly exposed to 0.5 and 1.0 M DMSO at 1 – 5 °C for 40 min. Cryoprotectant solutions were removed by two-step dilution with NSW as described for embryo experiments.

Salinity tolerance experiment

A hypersaline stock solution was prepared by dissolving NaCl in NSW to 250 ppt. Experimental saline solutions at 35, 55, 100, 150 and 200 ppt were prepared and cooled to 12 °C. Embryo exposure to saline solutions was similar to the protocol for cryoprotectants.

Parameters and statistics

At the start of each experiment, uncooled samples (four replicates) were incubated as an indicator of spawn quality (control 27 °C).. For all cooling, cryoprotectant, and salinity experiments, survival rate was estimated from four replicates of 70 – 100 embryos taken from one female in each experiment at each sample point. Survival of embryos was assessed by their ability to develop into active nauplius larvae after 16 h from spawning. Nauplii survival was evaluated by their

ability to recover normal activity after reaching ambient temperature (27 °C), for four replicates of 40 – 60 nauplii each.

Survival rates, expressed as %, were arcsine transformed to make the variance independent of the mean (Leung-Trujillo and Lawrence, 1987) and statistically analyzed for significant differences among the means, using one way classification analysis of variance (AOV) and Duncan's multiple range test (Ott, 1984); P values < 0.05 were regarded as significant. Figures present untransformed data as mean percentages ± standard error (s.e.).

Results

Fig. 1 shows the survival rate of *T. byrdi* embryos exposed at 10 °C for different time intervals; no statistical differences in survival were measured between embryos kept at 27 °C (0 min exposure time) and at 10 °C for as long as 180 min of exposure. Survival decreased statistically for both embryo stages after 240 min of exposure at 10 °C, being more dramatic for advanced embryos. Morulae and advanced embryos were very sensitive to 0 °C exposure, generating 100% mortality at 30 min and 60 min, respectively.

Cooling tolerance in *T. byrdi* embryos was also observed for each cryoprotectant tested and the salinity experiment (Figs. 2, 3, 4, 5 and 6). In each one of these experiments the control group at 27°C gave survival rates similar to morulae and advanced embryos cooled for 20 min at 12 °C without cryoprotectant (0 M); in the DMSO experiment (Fig. 2), cooled morulae at 0 M DMSO showed improved survival ($P < 0.05$) compared to the control group at 27 °C.

T. byrdi morulae showed a reduced survival rate as the DMSO concentration increased (Fig. 2); however, the standard deviation increased dramatically as compared to 0 M DMSO. A statistically significant reduction in survival was obtained for 1 M DMSO, and total mortality at higher concentrations. Advanced embryos exposed to 0.5 M DMSO showed no statistical difference in survival as compared to 0 M DMSO. However, survival was significantly reduced at 1 M DMSO, while 2 M DMSO generated high mortality (98.8%).

The *T. byrdi* embryo response to sucrose was similar for both stages (Fig. 3). Concentrations over 0.25 M sucrose gave a significant decrease in survival ($P < 0.05$) as compared to 0 M, for morulae and advanced embryos; total mortality occurred with sucrose concentration over 1 M.

T. byrdi advanced embryo exposure to methanol (Fig. 4) showed high survival at all concentrations tested, not different from 0 M ($P > 0.05$). Morula embryos could not be sampled on time in this experiment. Glycerol was very toxic to *T. byrdi* embryos

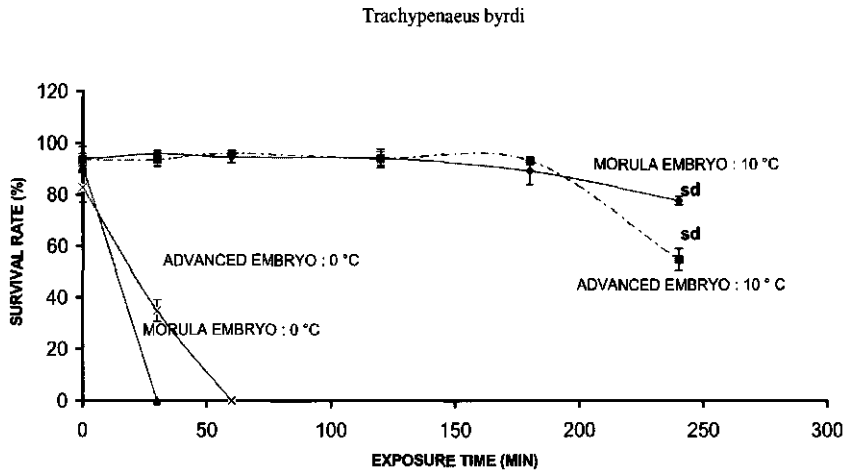

Figure 1. Survival of *T. byrdi* embryos exposed to low temperature in one-step cooling protocol from ambient temperature (27 °C). sd = statistically significant difference ($P < 0.05$).

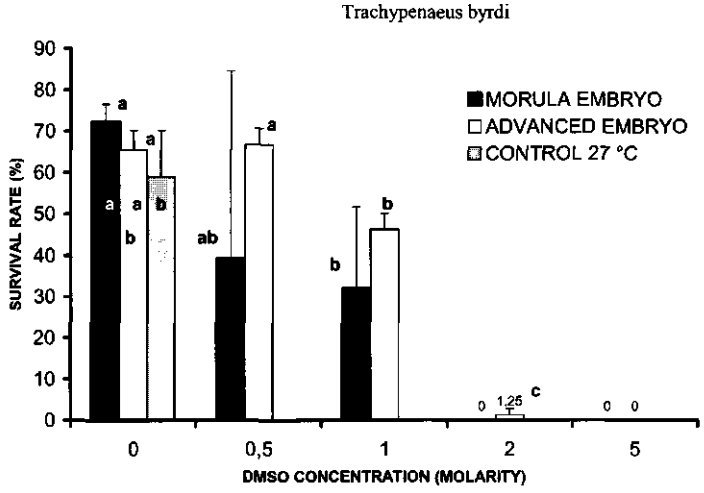

Figure 2. Survival of *T. byrdi* embryos exposed to dimethyl sulfoxide (DMSO) for 20 min at 12 °C. Bars and lines represent treatment means and standard errors, respectively. Bars with the same outside letters are not statistically different ($P > 0.05$). Bars with the same inside letters are not statistically different from control 27 °C.

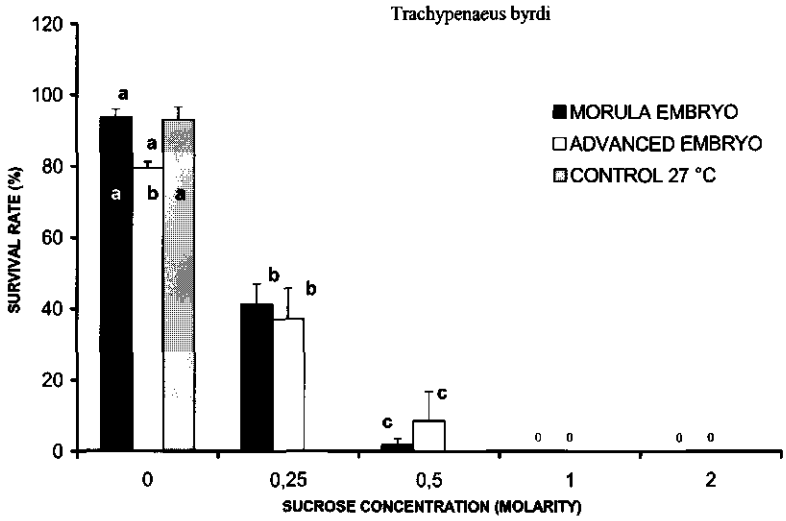

Figure 3. Survival of *T. byrdi* embryos exposed to sucrose for 20 min at 12 °C. Bars and lines represent treatment means and standard errors, respectively. Bars with the same outside letters are not statistically different (P > 0.05). Bars with the same inside letters are not statistically different from control 27 °C.

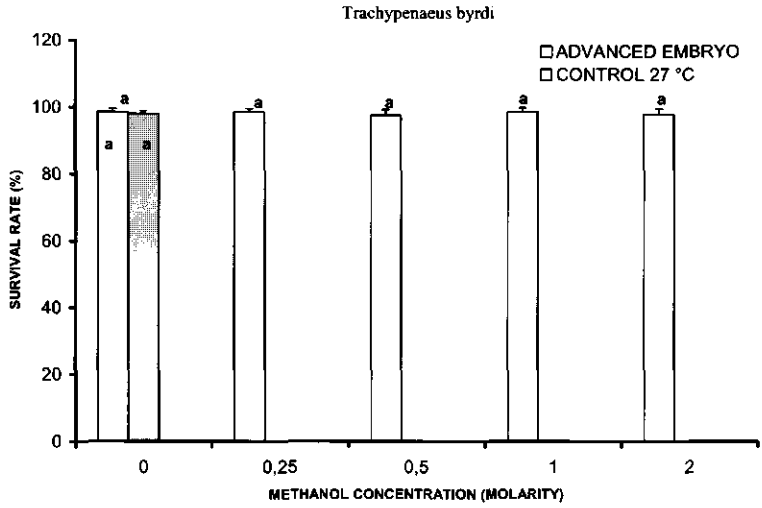

Figure 4. Survival of *T. byrdi* embryos exposed to methanol for 20 min at 12 °C. Bars and lines represent treatment means and standard errors, respectively. Bars with the same outside letters are not statistically different (P > 0.05). Bars with the same inside letters are not statistically different from control 27 °C.

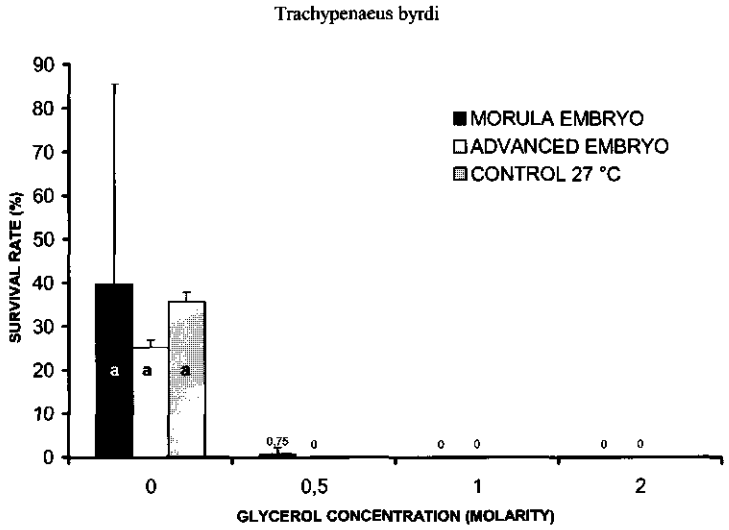

Figure 5. Survival of *T. byrdi* embryos exposed to glycerol for 20 min at 12 °C. Bars and lines represent treatment means and standard errors, respectively. Bars with the same outside letters are not statistically different ($P > 0.05$). Bars with the same inside letters are not statistically different from control 27 °C.

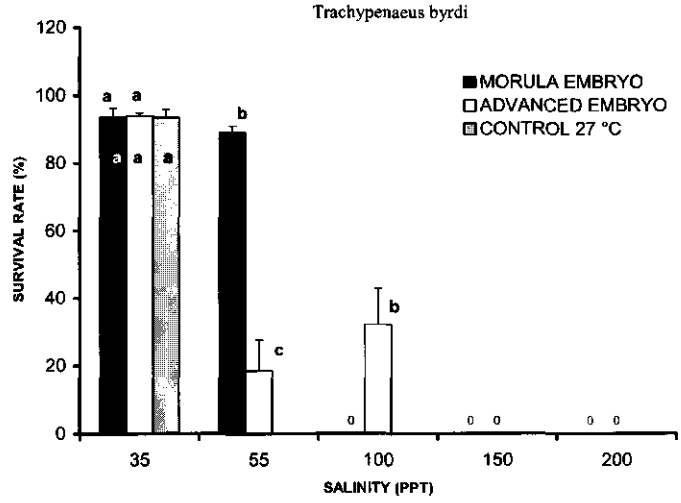

Figure 6. Survival of *T. byrdi* embryos exposed to hypersaline solutions for 20 min at 12 °C. Bars and lines represent treatment means and standard errors, respectively. Bars with the same outside letters are not statistically different ($P > 0.05$). Bars with the same inside letters are not statistically different from control 27 °C.

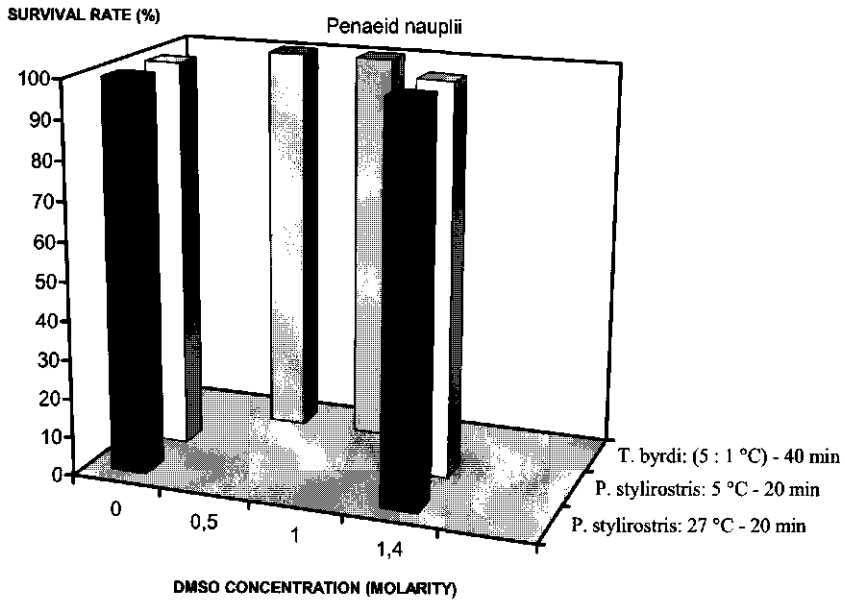

Figure 7. Survival of *P. stylirostris* and *T. byrdi* nauplii at low density (50 nauplii ml^{-1}) exposed to low temperature and dimethyl sulfoxide (DMSO). Bars represent treatment means; standard error is zero for each bar.

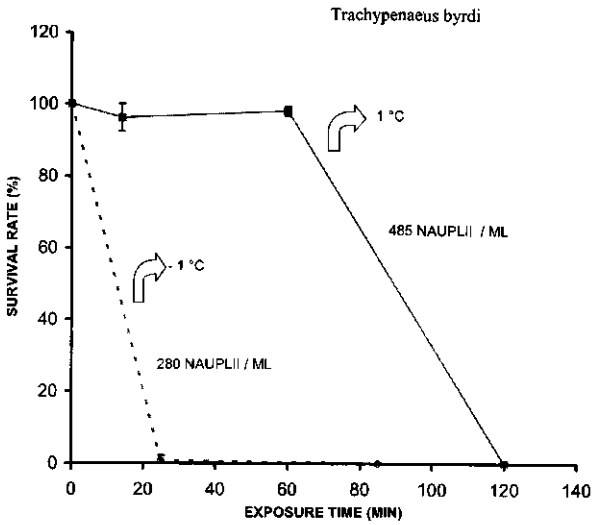

Figure 8. Survival of *T. byrdi* nauplii at high density exposed to low temperatures in one-step protocol.

(Fig. 5); generating almost total mortality at 0.5 M and higher concentrations; however, controls also had reduced survival related to the other experiments.

The salinity experiment (Fig. 6) show a low but statistically significant ($P < 0.05$) decrease in survival in *T. byrdi* morulae exposed at 55 ppt as compared to 35 ppt; at 100 ppt and higher salinities, 100% of morula embryos were damaged. Advanced embryos exhibited a more dramatic decrease in survival at 55 ppt than 100 ppt ($P < 0.05$); total mortality was observed at salinities over 150 ppt.

In general, nauplii larvae showed a better tolerance to cooling and DMSO exposure than embryos; for *P. stylirostris* the survival was 100% for larvae kept at 27 °C and 5 °C for 20 min (Fig. 7); the presence of 1.4 M DMSO did not affect survival rate. *T. byrdi* nauplii showed 100% survival when exposed from 5 to 1 °C during 40 min, in the presence of either 0.5 or 1.0 M DMSO. The maximum exposure time at 1 °C in *T. byrdi* nauplii at high density is 60 min (Fig. 8). On the contrary, exposure to –1 °C generated total mortality in less than 25 min.

Discussion

The developmental stage of *T. byrdi* embryos is crucial for cooling tolerance. In a previous experiment total mortality was obtained with 16-32 cell embryos cooled at 12 °C (uncooled control = 98.5% survival; unpublished results). Later stages of *T. byrdi* embryos tolerated cooling to 10 °C, but lower temperatures near 0 °C, caused high mortality. Morulae were more resistant to cooling at 10 °C than advanced embryos, but only advanced embryos tolerated exposure to 0 °C during 30 min (survival rate = 35 ± 4 %). Moreover, cooling to 0 °C in two steps (>12°C > 0 °C) did not improve embryo survival (unpublished observations). Based on these experimental data, direct cooling to 10 °C has no negative effect on embryo development; on the contrary, it seems that such a treatment may improve morulae survival in low quality spawns; the glycerol experiment was performed with a low quality spawn, as can be seen from the uncooled group survival (35.8 ± 2.2 %); this low quality appeared to improve for cooled morulae without glycerol, based on the high measured standard deviation. However, this hypothesis requires further confirmation. Nauplii also were more resistant to near 0 °C exposure than embryos; even at high density (485 nauplii ml^{-1}), they tolerated 60 min without a decrease in survival, but at –1 °C nauplii were extremely sensitive. In conclusion, early embryonal stages (16-32 cells) of *T. byrdi* are highly sensitive to cooling at 12 °C, while later stages (morulae and advanced embryos) are tolerant to 10 °C, and nauplii are resistant to 1 °C exposure.

It has been suggested that resistance to cooling is associated with lipid composition. Large amounts of lipid material in some mammalian embryos (pig, sheep and cow) and a temperature-induced phase change in the lipids during cooling, were

suggested to be responsible for destroying the physical integrity of the early embryonic cells (Whittingham, 1980). Oyster embryos (*Crassostrea gigas*) from low quality spawns were more susceptible to cooling than high quality ones, and the major ultrastructural feature of cooled oyster embryos was a loss of plasma membrane arrangement leading to a massive disruption of the microvilli from embryonic cells (Renard, 1991); the author suggested membrane-lipid sensitivity to cooling as the possible cause of microvilli disruption.

T. byrdi morulae and advanced embryos were more resistant to cooling than porcine, early stages of bovine, and oyster embryos (Wilmut, 1972; Wilmut et al., 1975; Renard, 1991). Early embryonal stages (2-4 cells) of oysters were sensitive even at 16 °C exposure, and at 5 °C exposure, survival was dramatically reduced (Renard, 1991).

Decapod crustaceans produce heavily yolked eggs, which accumulate phospholipid droplets containing polyunsaturated fatty acids (PUFAs; Harrison, 1990; Ravid et al., 1999). These lipid classes are associated with the survival of embryos and initial larval stages of marine shrimps; crustacean phospholipids generally contain low ω-6 but high ω-3 levels of PUFAs (Eicosapentaenoic acid 20:5ω-3 and Docosahexaenoic acid 22:6ω-3; Harrison, 1990); on the other hand, PUFAs from mammals are made by ω-6 fatty acids (Chapelle, 1986). The high levels of these two fatty acids in marine animals is believed to be related to the effective unsaturation of the melting point of a lipid; the ω-3 structure allows a greater degree of unsaturation than the ω-6 or ω-9, so that crustacean phospholipids permit a better flexibility of membrane structure at lower temperatures (Chapelle, 1986).

It is possible that the specific composition of embryo lipids affects the cooling sensitivity, so that omega-3 PUFAs present in penaeid and oyster (Labarta et al., 1999) eggs, but not in mammals, make embryos (late stages) more resistant to cooling. However, 16-32 cell *T. byrdi* embryos were extremely sensitive to 12 °C exposure; therefore, this sensitivity may be caused by factors other than lipid vesicles in early embryonal stages of marine invertebrates.

The longer-term tolerance to 10 °C exposure of late stage *T. byrdi* embryos is an advantage for developing a cryogenic protocol, since a major concern for freezing shrimp embryos is the time needed for permeation of cryoprotectant (Subramoniam and Arun, 1999), which may be accelerated by electroporation or cavitation (Bart and Zohar, 1998; Preston et al., 2000). Exposures to cryoprotectants are generally toxic. However, embryo exposure to equimolar solutions of cryoprotectants at 12 °C suggest that methanol is not toxic to advanced embryos, up to 2 M, although higher concentrations should be evaluated. DMSO was intermediate in terms of toxicity; advanced embryos tolerated 0.5 M DMSO without statistical reduction in survival, and

morulae showed variable survival within replicates. On the other hand, sucrose and glycerol were quite toxic to both embryo stages over 0.5 M and 0.25 M, respectively. For sucrose, a lower toxic level was detected based on concentrations evaluated; however, the sensitivity to glycerol was similar.

Fish embryos (Robertson et al., 1988) and oyster embryos (Renard, 1991) are reported to be sensitive to glycerol and sucrose, and more tolerant to DMSO and methanol. *P. indicus* morulae were also sensitive to glycerol (0.67 M), more tolerant to DMSO and methanol, and highly resistant to ethylene glycol (2.7 M; Subramoniam and Arun, 1999, for review). In general terms, it seems that *T. byrdi* embryos are more sensitive to cryoprotectants than *Sciaenops ocellatus* embryos (Robertson et al., 1988); however, methanol gave similar responses in both studies. The pattern of cryoprotectant tolerance found for *C. gigas* embryos was similar for *T. byrdi* embryos: methanol (high) > DMSO > glycerol > sucrose (low). The sensitivity pattern to glycerol, DMSO, and methanol was also similar between *P. indicus* and *T. byrdi* embryos; therefore, it seems that cryogenic protocols for penaeid embryos should be based on methanol or DMSO.

Penaeid nauplii are more resistant to DMSO exposure than embryos. *P. stylirostris* tolerated 1.4 M at ambient temperature and 5 °C, without survival compromise, for 20-min exposure time. *T. byrdi* nauplii showed excellent survival at 1 M DMSO, during 40-min exposure time. *T. byrdi* and *P. stylirostris* nauplius stage III-IV seems to tolerate higher DMSO concentrations; however, *P. monodon* nauplius stages IV and V-VI, generated 50% mortality at 2.8 M (20%) DMSO, after 20-min exposure time (Subramoniam and Arun, 1999, for review).

Hypersaline treatment at 12 °C indicates that morulae are more resistant than advanced embryos to 55 ppt exposure; this limited tolerance of morulae to hypersaline conditions makes the application of dehydrating embryos without exposure to toxic substances (Robertson et al., 1988) or combining hypersaline dehydration plus cooled exposure to selected cryoprotectants doubtful. Advanced embryos tolerated a higher salinity (100 ppt) than morulae, but advanced embryo sensitivity to a direct increment of 20 ppt indicates that future attempts to improve hypersaline tolerance should focus on reducing the salinity increments by which the final salinity is reached. However, Robertson et al. (1988) found no advantage of hypersaline serial dosing over direct plunge for *S. ocellatus* embryos.

Although in this study penaeid nauplii were more resistant to cooling and cryoprotectant exposure than advanced embryos, the less complex organization of an embryo and the qualities found in this research, indicate that cryopreservation trials with penaeid embryos could be successful.

Acknowledgements

This study was supported by Ley del Atún, No. 6267. The authors thank Gerardo Zúñiga and Captain Torres for their constant cooperation. To Dr. Jorge Gunther for manuscript review, and to unknown referees for their valuable comments. The results presented are part of the PhD thesis of J. Alfaro.

References

Alfaro, J., 1999. Resistencia al congelamiento y criopreservación de embriones, larvas y semen de organismos acuáticos. Rev. Invest. Mar. 20 (1-3), 73 - 80.

Anchordoguy, T., Crowe, J. H., Griffin, F. J., Clark, W. H., Jr., 1988. Cryopreservation of sperm from the marine shrimp *Sicyonia ingentis*. Cryobiology 25, 238 – 243.

Asahina, E., Takahashi, T., 1979. Cryopreservation of sea urchin embryos and sperm. Dev. Growth Differ. 21 (5), 423 – 430.

Bart, A., Zohar, Y., 1998. Cryopreservation of fish embryos: current status. Annual meeting of the World Aquaculture Society. Las Vegas, USA, 15 – 19 February 1998. Abstract.

Baust, J. G., Lawrence, A. L., 1977. Rapid freezing: an essential criterion for the successful cryopreservation of immature larval shrimp. Cryobiology 14, 705.

Benzie, J. A. H., 1998. Penaeid genetics and biotechnology. Aquaculture 164, 23 – 47.

Browdy, C., 1992. A review of the reproductive biology of *Penaeus* species: perspectives on controlled shrimp maturation systems for high quality nauplii production. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 22 – 51.

Chapelle, S., 1986. Aspects of phospholipid metabolism in crustaceans as related to changes in environmental temperatures and salinities. Comp. Biochem. Physiol. 84B (4), 423 – 439.

Chow, S., Taki, Y., Ogasawara, Y., 1985. Cryopreservation of spermatophore of the fresh water shrimp, *Macrobrachium rosenbergii*. Biol. Bull. 168, 471 – 475.

Clegg, J. S., Willsie, J. K., Jackson, S. A., 1999. Adaptive significance of a small heat shock/alpha –crystallin protein (P26) in encysted embryos of the brine shrimp, *Artemia franciscana*. Amer. Zool. 39, 836 – 847.

Clifford, H., 1992. Marine shrimp pond management: a review. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 110 – 137.

Gallardo, C. S., Del Campo, M. R., Filun, L., 1988. Preliminary trials of the cryopreservation of marine mollusk embryos as illustrated with the marine mussel *Choromytilus chorus* from Southern Chile. 25th Meeting of the Society for Cryobiology. Aachen 11 – 15 July 1988. Abstract 164.

Green, J., 1965. Chemical embryology of the crustacea. Biol. Rev. 40, 580 – 600.

Harrison, K. E., 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: a review. J. Shellfish Res. 9 (1), 1 – 28.

Khin-Maung, O., Kurokura, H., Iwano, T., Okamoto, K., Kado, R., Hino, A., 1998. Cryopreservation of nauplius larvae of the barnacle, *Balanus amphitrite* Darwin. Fish. Sci. 64 (6), 857 – 860.

Kungvankij, P., Tiro, L. B., Pudadera, B. J., Jr., Potestas, I. O., Corre, K. G., Borlongan, E., Talean, G. A., Bustilo, L. F., Tech, E. T., Unggui, A., Chua, T. E., 1986. Shrimp hatchery design, operation and management. Network of Aquaculture Centers in Asia, Philippines, 88 pp.

Labarta, U., Fernández-Reiriz, M. J., Pérez-Camacho, A., 1999. Dynamics of fatty acids in the larval development, metamorphosis and post-metamorphosis of *Ostrea edulis* (L.). Comp. Biochem. Physiol. 123A, 249 – 254.

Leung-Trujillo, J. R., Lawrence, A. L., 1987. Observations on the decline in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65, 363 – 370.

McFadzen, I. R. B., Utting, S. D., 1990. The viability and growth of cryopreserved bivalve larvae. Annual meeting of the World Aquaculture Society. Halifax, Canada, 10 – 14 June 1990. Abstract T13.6.

Nowshari, M. A., Brem, G., 1998. Effect of cryoprotectants and their concentration on post-thaw survival and development of expanded mouse blastocysts frozen by a simple rapid-freezing procedure. Theriogenology 50, 1001 – 1013.

Ott, L., 1984. An introduction to statistical methods and data analysis. Duxbury Press, Boston, Massachusetts, USA, 775 pp.

Preston, N. P., Baule, V. J., Leopold, R., Henderling, J., Atkinson, P. W., Whyard, S., 2000. Delivery of DNA to early embryos of the kuruma prawn, *Penaeus japonicus*. Aquaculture 181, 225 – 234.

Ravid, T., Tietz, A., Khayat, M., Boehm, E., Michelis, R., Lubzens, E., 1999. Lipid accumulation in the ovaries of a marine shrimp *Penaeus semisulcatus* (De Haan). J. Exp. Biol. 202, 1819 – 1829.

Renard, P., 1991. Cooling and freezing tolerance in embryos of the pacific oyster, *Crassostrea gigas*: methanol and sucrose effects. Aquaculture 92, 43 – 57.

Robertson, S. M., Lawrence, A. L., Neil, W. H., Arnold, C. R., McCarty, G., 1988. Toxicity of the cryoprotectants glycerol, dimethyl sulfoxide, ethylene glycol, methanol, sucrose, and sea salt solutions to the embryo of red drum. Prog. Fish Cult. 50, 148 – 154.

Samocha, T. M., Lawrence, A. L., 1992. Shrimp nursery systems and management. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 87 - 105.

Subramoniam, T., Arun, R., 1999. Cryopreservation and aquaculture: a case study with penaeid shrimp larvae. Curr. Sci. 76 (3), 361 – 368.

Treece, G. D., Yates, M. E., 1988. Laboratory manual for the culture of penaeid shrimp larvae. TAMU-SG-88-202, Texas A and M University, USA, 95 pp.

Whittingham, D. G., 1980. Principles of embryo preservation. In: Ashwood-Smith, M. J. and Farrant, J. (Eds.), Low temperature preservation in medicine and biology, University Park Press, Maryland, USA, pp. 65 – 84.

Wilkenfeld, J. S., 1992. Commercial hatchery status report: an industry panel viewpoint. In: Wyban, J. (Ed.), Proc. of the Special Session on Shrimp Farming, World Aquaculture Society, Baton Rouge, LA, USA, pp. 71 - 83.

Wilmut, I., 1972. The low temperature preservation of mammalian embryos. J. Reprod. Fertil. 31, 513 – 514.

Wilmut, I., Polge, C., Rowson, L. E. A., 1975. The effect on cow embryos of cooling to 20, 0 and –196 °C. J. Reprod. Fertil. 45, 409 – 411.

Chapter 9

General discussion

Introduction

The general objective of this research was to contribute to the understanding of penaeid reproduction and to explore new techniques for controlled shrimp reproduction. In the first paragraph we discuss the consequences of our findings on natural reproduction of shrimp in the Gulf of Nicoya for the shrimp fishery industry in Costa Rica. Controlled breeding of shrimp can benefit the shrimp farming industry in many ways. However, techniques to obtain good quality of gametes, and protocols for *in vitro* fertilization are needed. In the second paragraph several aspects of spermatophore quality, and ways to improve this, are discussed. In the third paragraph the implications of serotonin as an alternative to eyestalk ablation for maturation and spawning of females are discussed. In the fourth paragraph the possibilities of *in vitro* fertilization for shrimp culture are explored. Once *in vitro* fertilization is developed, techniques for cryopreservation of gametes and embryos can be developed. The last paragraph deals with the uses and prospects of cryopreservation of shrimp embryos.

Shrimp reproduction in Gulf of Nicoya

In recent years, our understanding about shrimp reproduction in the Gulf of Nicoya has increased remarkably. Catches from the artisanal shrimp fishery in the inner section of Gulf of Nicoya contain *P. occidentalis* (56.47% of total captures), *P. stylirostris* (31.72%), and *Trachypenaeus byrdi* (11.81%; Angulo, 1993).

These species mature and spawn throughout the year, with some peaks: *P. occidentalis* and *P. stylirostris* show a peak in reproduction activity between August and October, and another one between March and June, corresponding with the transition from the dry to the rainy season (Angulo, 1993; Tabash and Palacios, 1996); *T. byrdi* shows a reproduction peak between July and August, and again between December and February (Angulo, 1993; Castro, 1999). Other penaeid species found in this tropical estuary are *P. vannamei* (few specimens were captured), *T. faoea*, and *Xiphopenaeus riveti*.

The reproductive biology of *Penaeus occidentalis* in Gulf of Nicoya indicates that this species finds an adequate environment in the interior of this estuary for propagation. This zone is shallow, less than 20 m deep, and surrounded by mangroves. The previous believe was that the inner gulf did not serve as reproductive grounds for penaeid shrimps, but results presented in Chapter 2 show a normal reproductive activity in that area. Females of *P. occidentalis* mature and mate in this zone, then spawning occurs and eggs are released to start a new biological cycle. In nature, these events happen periodically, during which about 150,000 eggs per female are released. Wild *P. stylirostris* matured in captivity (Pizarro and Alfaro, 1994) produce an average of 220,000 eggs, and *P. vannamei* produce around 200,000 eggs (Chapter 7).

P. occidentalis is very sensitive to captivity. Eyestalk ablation does not induce ovarian maturation in wild females (Alfaro, unpublished data), and wild impregnated females remove their sperm mass after capture leading to infertile spawns. Moreover, manual ejaculation induces lethal damages to ampoules and vas deferens. These characteristics must be taken into account when considering the cage culture potential as an alternative to fisheries, as has been reported for *P. stylirostris* (Martínez-Córdova, 1988) and *P. vannamei* (Paquotte et al., 1998).

The Gulf of Nicoya is supporting a great pressure from artisanal fishery. The current understanding of shrimp reproduction in the Gulf of Nicoya allows a better implementation of regulatory measurements for stock sustainability. Shrimp stocks should be protected during reproductive peaks, and particular areas of intense reproductive activity must be closed to the fisheries. Penaeid species must be reproducing along the coastal waters of Central America, but there is not much scientific information available from other coastal areas in the region. Therefore, the Gulf of Nicoya is just a starting point. Reproductive biology studies from other coastal regions are needed so that a more integrated approach can be applied for the protection of our wild populations.

Spermatophore quality

The studies presented in this thesis clearly demonstrate that male shrimp are affected in their reproductive performance in captivity. The severity and nature of the problem are different between species.

Open thelycum species present high numbers of sperm per compound spermatophore in nature, as was found for *P. setiferus* from Gulf of Mexico (45 million sperm: Alfaro, 1990), and *P. occidentalis* from Gulf of Nicoya (49.5 million sperm: Chapter 2). However, closed thelycum species, having smaller spermatophores, present lower sperm counts, as was measured in the female seminal receptacles of *T. byrdi* from Gulf of Nicoya (6.8 million sperm: Alfaro, unpublished data), and *P. monodon* single spermatophores (one half) from Gulf of Thailand (2.7 million sperm: Pratoomchat et al., 1993).

Male penaeid shrimp are susceptible to fertility problems. The interaction between Male Reproductive Blackening Disease (MRBD) and bacterial infection, and the controlled induction of the disease in apparently healthy animals by gonopore injection of the bacteria, allowed to establish a link to the possible cause of MRBD. Previous studies considered 2 infertility problems of *P. setiferus* as the same disease process, namely, MRBD and Male Reproductive Tract Degenerative Syndrome (MRTDS). However, the results presented in this thesis revealed that there may be two different phenomena, without ruling out the possibility of one progressive syndrome, with a bacterial infection becoming involved in the advanced stages. Similar signs were

observed in some pond-grown *P. vannamei* in a maturation facility, and bacteria were also recovered from black reproductive sections (Alfaro, unpublished data). Following the publication of these results (Alfaro et al., 1993), Pascual et al. (1998) confirmed the different nature of MRBD and MRTDS. Their study showed that a water temperature between 24 and 27 C° reduces the incidence of reproductive tissue melanization and spermatophore degeneration in *P. setiferus*.

Based on our findings, an interpretation was proposed to explain the occurrence of MRBD in *P. setiferus*. Some opportunistic bacteria, of at least three different genera, penetrate the ampoule through gonopores. Micro-organisms reach the surface of spermatophore acellular matrix and vas deferens, where black depositions appear. They find a good supporting chitinous substrate for their propagation, and the shrimp's defense system is activated. Melanin, which is a defense and toxic molecule, is produced by the shrimp's prophenoloxidase system. Pascual et al. (1998) applied these findings about MRBD using localized erythromycin treatment after electrostimulation in order to prevent infection by opportunistic bacteria and they suggested that the treatment is effective.

Our observations on *P. stylirostris* and those in previous reports (Chapter 4) on spermatophore quality, lead us to propose a model with three independent levels of physiological control for male maturation: testes maturation, vas deferens maturation, and spermatophore synthesis. This would mean that the final quality of a spermatophore may be affected by the stage of development, the environment, and the nutrition, which by unknown mechanisms act at one or more of these physiological levels. This model was proposed to explain experimental data in the sense that the common parameters, measured to evaluate the quality of a spermatophore (sperm count, sperm abnormality, and spermatophore size/appearance), follow independent patterns. In other words, a high sperm count is not directly associated with low abnormalities, or a spermatophore with good appearance is not directly associated with adequate sperm counts or low abnormalities.

Similar unrelated responses in sperm counts, abnormalities, and spermatophore weights were measured in *P. vannamei.* Pond-grown males had improved sperm counts and reduced sperm abnormalities during laboratory culture. Eyestalk ablation had a measurable effect on sperm counts and spermatophore weight, but not on abnormalities, during the experimental period (Chapter 5). Recently, we evaluated *P. vannamei* spermatophores from a commercial facility, showing a good ratio of normal sperm cells, but low sperm counts (Alfaro, unpublished data).

A recent study from Ecuador indicates that wild *P. vannamei*, 2.5 to 3 months after capture, show sperm counts in the range of 13 million sperm per compound spermatophore (Wouters et al., 1999). Similar levels were measured by Wang et al.

(1995) for pond-grown *P. vannamei*, whereas Leung-Trujillo and Lawrence (1985) reported 30 million sperm also for pond-grown *P. vannamei*, and Wouters et al. (1999) obtained high sperm counts (28.7 million) in one of their experimental treatments. This seems to corroborate the before mentioned pattern in the sense that controlled reproduction of this species is obviously performed with different quality brooders, due to differences in local husbandry management and diet composition.

Spermatophore deterioration in *P. vannamei* was proposed as a normal process in penaeid shrimp reproduction (Chapter 5). Once a compound spermatophore is produced, it will be viable for a given period of time, then it may deteriorate until a residue is formed, while a new compound spermatophore is being produced. This deterioration process is completely different from that observed in *P. setiferus* (Alfaro, 1990), where the process involves the complete reproductive system, generating infertile males after 35 days of captivity. It is possible that both mechanisms are physiologically related, but that in *P. setiferus* the stress associated with captivity generates an unnatural response.

In conclusion, our studies about spermatophore quality in *P. setiferus*, *P. occidentalis*, *P. stylirostris*, and *P. vannamei* indicate that captivity induces a decrease in sperm quality for mature males from the wild, affecting sperm counts but not sperm abnormalities. However, young males from the wild or farm culture show high rates of immature sperm cells (spikeless cells), and adequate husbandry techniques (nutrition) can improve spermatophore quality when such males are subsequently raised in maturation tanks. Our studies also show that ejaculation and spermatophore regeneration have a positive effect on sperm maturation, and that in some species, spermatophores degenerate within ampoules as a normal process for renewal. Melanization or blackening of the reproductive system and infertility may develop in stressed or gonopore-damaged males, e.g. after manual or electrical ejaculation. The dramatic infertility by deterioration of the reproductive system in wild *P. setiferus* is however still poorly understood.

To improve shrimp reproduction, male broodstock have to be constantly monitored for melanization of reproductive tissue, natural deterioration of spermatophores, and male population status in sperm counts and abnormalities. By taking this information into account, commercial and academic facilities may be able to improve their husbandry management.

Quality of spermatophores from pond-grown males (*P. vannamei*) can also be improved by 17-alpha-methyltestosterone injection at 0.01 and 0.1 $\mu g\ g^{-1}$ body weight (Chapter 6). The improvement was evidently significant, but the precise physiological role of this compound has not yet been identified for decapod crustaceans (Quackenbush, 1986; Fingerman, 1987; 1997). In *P. monodon*, methyltestosterone also

enhanced spermatogenesis at 0.1 μg g^{-1} body weight (Yashiro et al., 1998). The induction could be the result of a pharmacological effect, activating spermatogenesis or spermatophore synthesis by high doses of non-physiologically related molecules, or it could be that 17-alpha-methyltestosterone is structurally related to the putative androgenic gland hormone responsible for the induction of spermatogenesis or spermatophore synthesis.

These studies, one in an open thelycum shrimp (Chapter 6) and the other in a closed thelycum shrimp (Yashiro et al., 1998), are just the starting point in this line of research. To further explore this subject, and to evaluate its practical application in shrimp mariculture, more research is recommended.

A novel approach for maturation and spawning in females

Eyestalk ablation is a non-reversible technique, which leads to a loss in egg quality and eventually death (Benzie, 1998). Moreover, it has been pointed out that nauplii production is supported by a low proportion of ablated females, while the rest of the ablated population generates few spawnings (Bray et al., 1990). Palacios et al. (1999a) suggested selection on multiple spawning capability in females from pond-reared broodstock to improve nauplii production. However, spawner exhaustion will still occur over time after ablation (Palacios et al., 1999b).

Serotonin injection in wild female *P. vannamei* induced ovarian maturation and spawning, although at a lower rate than unilateral eyestalk ablation (Chapter 7). So far these results are the best, published alternative treatment for shrimp maturation. Recently, methyl farnesoate (MF) has been reported to increase fecundity in cultured *P. vannamei* (Laufer et al., 1997). The following hypotheses were proposed to explain the lower (i.e. compared to ablation) effect of serotonin on *P. vannamei* maturation:

a) Gonad inhibiting hormone control over ovaries and hepatopancreas is very intense.
b) Elevated glucose levels induced by serotonin injection have a negative effect on maturation.
c) The inhibition of MF synthesis by serotonin injection has a negative effect on maturation.

The third hypothesis may be the most limiting obstacle to higher maturation rates because MF elevations are required for stimulating ovarian maturation in crustaceans (Que-Tae et al., 1999). These authors suggested that concentrations of MF in the hemolymph regulate both the initiation of ovarian development and also the rate at which maturation takes place.

Hypothetically, females treated with serotonin should be capable of a longer reproductive period since reproductive exhaustion may be controlled through an

adequate protocol. However, a single molecular approach does not seem to be an acceptable alternative to eyestalk ablation, due to the multifactorial regulation of female shrimp reproduction, as indicated by Huberman (2000). Based on the present understanding of crustacean endocrinology, a "combined molecular approach", e.g. injecting serotonin and MF, should be explored in the near future.

***In vitro* fertilization**

Genetic manipulations of shrimp such as polyploidy induction, hybridization, and genetic engineering, are only possible when gametes can be handled under *in vitro* conditions. The present understanding of gamete interaction amongst the Dendrobranchiata is restricted to selected species of penaeids (Clark et al., 1984; Shigekawa and Clark, 1986; Pillai et al., 1988). *In vitro* induction of the acrosome reaction is a practical way to evaluate sperm capacitation and sperm viability in penaeids. Griffin et al. (1987) developed a technique to induce the acrosome reaction, using the jelly precursor released by eggs during spawning in artificial seawater, contained in beakers (the egg water technique). The penaeid jelly precursor contains 75% protein and 25-30% carbohydrate; proteases, antibacterial agents, and acrosome reaction inducers are suggested as constituents (Lynn and Clark, 1987). The technique was applied in *S. ingentis*, generating 75% reactive sperm by 5 min exposure. Anchordoguy et al. (1988) used the same technique to assess viability of *S. ingentis* cryopreserved sperm (56% survival). Later, the egg water technique was also used for sperm analysis in *P. monodon* (Pratoomchat et al., 1993) and *P. vannamei* (Wang et al., 1995). The first study reported 4.2% reactive sperm for wild-caught shrimp (body weight of 46 – 138 g; 2.7 million sperm/ spermatophore), and the second one reported 37.4% reactive sperm for pond-grown animals (body weight of 41.1 g; 13.1 million sperm/ spermatophore). However, our observations about *in vitro* spawning of *P. occidentalis* showed no acrosome reaction, what so ever. The acrosome reaction of sperm cells in suspension, was not adequately activated by the egg jelly in this open thelycum species. This could be the result of the protocol used for sperm homogenization, which separates sperm cells from their matrix.

In developing a technique for *in vitro* fertilization, the sperm : egg ratio is a valuable parameter to take into account, particularly for penaeid shrimp since it has been suggested that polyspermy may be necessary for fertilization (Clark et al., 1980). In *P. occidentalis*, a single female is impregnated by one male, who transfers about 50 million sperm cells. Based on these findings, it can be calculated that the sperm : egg ratio is around 333 : 1 for natural spawning which is rather low compared to other marine organisms such as fish. The *in vitro* fertilization protocols published so far are characterized by the preparation of a sperm suspension diluted in seawater, given the

following densities: 54,000 sperm ml^{-1} (Clark et al., 1973), 150,000 – 305,000 sperm ml^{-1} (Alfaro et al., 1993), and over 10^6 sperm ml^{-1} (Misamore and Browdy, 1997). However, no information on the sperm : egg ratio is given in these studies. The highest fertilization rate occurred at the lowest sperm density in *P. aztecus*, which is a closed thelycum species. However, these results have not been replicated (Bray and Lawrence, 1992). In *P. monodon*, a 50% hatching rate was reported (Lin and Ting 1984 cited in Primavera, 1985).

Based on the spermatophore differences we described between open and closed thelycum shrimp, it is proposed that a novel technical approach should be considered in the future. Open thelycum shrimp would require a high sperm : egg ratio at the moment of interaction to obtain an acceptable fertilization rate. Additionally, sperm suspensions should be avoided to imitate the natural fertilization mechanism, by which sperm cells are attached to a matrix, as is seen in the medial vas deferens of *P. stylirostris* by electron microscopy (Alfaro, 1994). The sperm matrix is fixed to the female thelycum, and eggs will be pumped through the matrix, allowing an instant primary binding between sperm spikes and vitelline envelopes. Since it has been demonstrated that the release of jelly precursors is a Mg^{+2}-dependent event (Clark and Lynn, 1977), spawning and mixing with sperm in Mg^{+2} free seawater before transferring to normal seawater may improve fertilization rates, but this hypothesis has to be investigated. Closed thelycum species, on the other hand, seem to require a lower sperm : egg ratio, and sperm suspensions proved to be acceptable for *in vitro* fertilization. Our suggestion is that this protocol is somehow similar to the natural mechanism since this group of shrimp keeps the sperm cells within the seminal receptacle, from where the cells are pumped to be mixed with released eggs.

Embryo qualities for cryopreservation

Shrimp embryos showed good tolerance to cooling, and the cooling tolerance increases with the developmental stage, with the nauplius larvae being the most resistant. Ethylene glycol and methanol are the most suitable cryoprotectants, so far tested, for developing cryogenic protocols with shrimp embryos. Hypersaline dehydration does not seem adequate for shrimp embryos since they proved highly sensitive to increased salt concentration.

Research on cold tolerance conducted in penaeid embryos (Chapter 8) has shown that morulae and advanced stages are resistant to 10 °C exposure without compromising survival. Methanol up to 2 M was not toxic to advanced embryos, and DMSO was tolerated at 0.5 M. In recent experiments, ethylene glycol was tested at 1.5 M and 15 min of exposure time without any effect on survival rate (own unpublished data). A 20 ppt increment in salinity is not detrimental to shrimp morulae, but 65 ppt

causes total mortality. Early embryos (16 – 32 cells) proved very sensitive to 12 °C exposure (100 % mortality); morulae and advanced embryos were very sensitive to sucrose and glycerol exposure.

Cold tolerance in shrimp embryos could be related to the presence of omega-3 PUFAs in penaeid eggs. A related hypothesis was offered by Hochi et al. (1999) to explain the positive action of linoleic acid-albumin on cryopreservation of pronuclear bovine zygotes. Penaeid eggs from the wild are particularly rich in PUFAs; however, under controlled reproduction the level of these fatty acids may decrease depending on nutrition and spawner exhaustion.

Penaeid embryos or larvae have not been cryopreserved yet, but this basic knowledge about cold tolerance and cryoprotectant toxicity will help to define cryogenic protocols. Freezing and vitrification of fish embryos have generated unsuccessful results so far and fish researchers are now more interested in the cryopreservation of fish blastomeres (Leveroni and Maisse, 1999). In crustaceans, the study on cryopreservation of the nauplius larvae of the barnacle, *Balanus amphitrite* (Khin-Maung et al., 1998), is encouraging. Moreover, the cryopreservation of *Artemia* embryos (Alfaro, 1999) is also a positive indicator for crustaceans, although *Artemia* is adapted to tolerate dryness and freezing through glycerol protection (Green, 1965), which allows hydrated cysts to withstand –18 °C in nature (Sorgeloos et al., 1986).

Cryopreservation of embryos will be of great benefit to the shrimp industry. This industry is currently relying on expensive international seedstock transportation, using high volumes of water to ensure larvae survival. Another immediate application would be the storage of embryos in reproduction facilities, where overproduction of embryos usually occurs at certain times of the year. From a genetic perspective, cryopreservation of embryos would favor the implementation of breeding programs and genetic manipulation.

References

Alfaro, J., 1990. A contribution to the understanding and control of the male reproductive system melanization disease of broodstock *Penaeus setiferus*. Master Thesis, Department of Wildlife and Fisheries Sciences, Texas A & M University, USA.

Alfaro, J., 1994. Ultraestructura de la glándula androgénica, espermatogénesis y oogénesis de camarones marinos (Decapoda: Penaeidae). Rev. Biol. Trop., 42, 121 – 129.

Alfaro, J., 1999. Resistencia al congelamiento y criopreservación de embriones, larvas y semen de organismos acuáticos. Rev. Invest. Mar. 20 (1-3), 73 – 80.

Alfaro, J., Palacios, J. A., Aldave, T. M., Angulo, R. A., 1993. Reproducción del camarón *Penaeus occidentalis* (Decapoda: Penaeidae) en el Golfo de Nicoya, Costa Rica. Rev. Biol. Trop. 41, 563 – 572.

Anchordoguy, T., Crowe, J. H., Griffin, F. J., Clark, W. H. Jr., 1988. Cryopreservation of sperm from the marine shrimp *Sicyonia ingentis*. Cryobiology 25, 238 – 243.

Angulo, R., 1993. Crecimiento, mortalidad y madurez gonadal de *Penaeus stylirostris*, *Penaeus occidentalis* y *Trachypenaeus byrdi* en el Golfo de Nicoya, Costa Rica. Tesis de Licenciatura, Escuela de Ciencias Biológicas, Universidad Nacional, Costa Rica.

Benzie, J. A. H., 1998. Penaeid genetics and biotechnology. Aquaculture 164, 23 – 47.

Bray, W. A., Lawrence, A. L., 1992. Reproduction of *Penaeus* species in captivity. In: Fast, A., Lester, L. J. (Eds.), Marine shrimp culture: principles and practices, Elsevier Science Publishers B. V., Amsterdam, The Netherlands, pp. 93 – 169.

Bray, W. A., Lawrence, A. L., Lester, L. J., 1990. Reproduction of eyestalk-ablated *Penaeus stylirostris* fed various levels of total dietary lipids. J. World Aquacult. Soc. 21, 41 – 52.

Castro, S. W., 1999. El camarón conchudo *Trachypenaeus byrdi* (Decapoda: Penaeidae): algunos aspectos de su biología y evaluación de la población alrededor de la Isla Chira, Golfo de Nicoya, Costa Rica. Tesis de Licenciatura, Escuela de Ciencias Biológicas, Universidad Nacional, Costa Rica.

Clark, W. H. Jr., Lynn, J. W., 1977. A Mg^{++} dependent cortical reaction in the eggs of penaeid shrimp. J. Exp. Zool. 200, 177 – 183.

Clark, W. H. Jr., Lynn, J. W., Yudin, A. I., Persyn, H. O., 1980. Morphology of the cortical reaction in the eggs of Penaeus aztecus. Biol. Bull. 158 (2), 175 – 186.

Clark, W. H. Jr., Talbot, P., Neal, R. A., Mock, C. R., Salser, B. R., 1973. *In vitro* fertilization with non – motile spermatozoa of the brown shrimp *Penaeus aztecus*. Mar. Biol. 22, 353 – 354.

Clark, W. H. Jr., Yudin, A. I., Griffin, F. J., Shigekawa, K., 1984. The control of gamete activation and fertilization in the marine Penaeidae, *Sicyonia ingentis*. In: Engels, W., Clark, W. H. Jr., Fisher, A., Olive, P. G. W., Went, D. F. (Eds.), Advances in Invertebrate Reproduction, vol. 3, Elsevier Science Publisher, Amsterdam, pp. 459 – 472.

Fingerman, M., 1987. The endocrine mechanisms of crustaceans. J. Crustacean Biol. 7 (1), 1 – 24.

Fingerman, M., 1997. Crustacean endocrinology: a retrospective, prospective, and introspective analysis. Physiol. Zool. 70 (3), 257 – 269.

Green, J., 1965. Chemical embryology of the crustacea. Biol. Rev. 40, 580 – 600.

Griffin, F. J., Clark, W. H. Jr., Crowe, J. H., Crowe, L. M., 1987. Intracellular pH decrease during the *in vitro* induction of the acrosome reaction in the sperm of *Sicyonia ingentis*. Biol. Bull. 173, 311 – 323.

Hochi, S., Kanamori, A., Sugisawa, K., Kimura, K., Hanada, A., 1999. Effect of linoleic acid-albumin in the IVM and IVF media on survival of frozen-thawed pronuclear bovine zygotes. J. Mamm. Ova Res. 16, 19 – 22.

Huberman, A., 2000. Shrimp endocrinology. A review. Aquaculture 191, 191 – 208.

Khin-Maung, O., Kurokura, H., Iwano, T., Okamoto, K., Kado, R., Hino, A., 1998. Cryopreservation of nauplius larvae of the barnacle, *Balanus amphitrite* Darwin. Fish. Sci. 64 (6), 857 – 860.

Laufer, H., Paddon, J., Paddon, M., 1997. A hormone enhancing larval production in the Pacific white shrimp *Penaeus vannamei*. In: Alston, D. E., Green, B. W., Clifford, H. C. (Eds.), IV Symposium on Aquaculture in Central America: focusing on shrimp and tilapia. The Latin American Chapter of the World Aquaculture Society, Tegucigalpa, Honduras, pp. 161 – 162.

Leung-Trujillo, J. R., Lawrence, A. L., 1985. The effect of eyestalk ablation on spermatophore and sperm quality in *Penaeus vannamei*. J. World Maric. Soc. 16, 258 - 266.

Leveroni, S. C., Maisse, G., 1999. Cryopreservation of carp (*Cyprinus carpio*) blastomeres. Aquat. Living Resour. 12 (1), 71 – 74.

Lynn, J. W., Clark, W. H. Jr., 1987. Physiological and biochemical investigations of the egg jelly release in *Penaeus aztecus*. Biol. Bull. 173, 451 – 460.

Martínez-Córdova, L., 1988. Culture of blue shrimp (*Penaeus stylirostris*) in floating cages. Prog. Fish Cult. 50, 36 – 38.

Misamore, M., Browdy, C., 1997. Evaluating hybridization potential between *Penaeus setiferus* and *Penaeus vannamei* through natural mating, artificial insemination and *in vitro* fertilization. Aquaculture 150, 1 – 10.

Palacios, E., Racotta, I. S., APSA, 1999a. Spawning frequency analysis of wild and pond-reared pacific white shrimp *Penaeus vannamei* broodstock under large-scale hatchery conditions. J. World Aquacult. Soc. 30 (2), 180 – 191.

Palacios, E., Perez-Rostro, C. I., Ramirez, J. L., Ibarra, A. M., Racotta, I. S., 1999b. Reproductive exhaustion in shrimp (*Penaeus vannamei*) reflected in larval biochemical composition, survival and growth. Aquaculture 171, 309 – 321.

Paquotte, P., Chim. L., Martin, J., Lemos, E., Stern, M., Tosta, G., 1998. Intensive culture of shrimp *Penaeus vannamei* in floating cages: zootechnical, economic and environmental aspects. Aquaculture 164, 151 – 166.

Pascual, C., Valera, E., Re-Regis, C., Gaxiola, G., Sanchez, A., Ramos, L., Soto, L. A., Rosas, C., 1998. Effect of water temperature on reproductive tract condition of *Penaeus setiferus* adult males. J. World Aquacult. Soc. 29 (4), 477 – 484.

Pillai, M. C., Griffin, F. J., Clark, W. H. Jr., 1988. Induced spawning of the decapod crustacean *Sicyonia ingentis*. Biol. Bull. 174, 181 – 185.

Pizarro, F., Alfaro, J., 1994. Reproductive performance of *Penaeus stylirostris* females injected with heat-killed *Vibrio alginolyticus*. J. World Aquacult. Soc. 25, 576 – 578.

Pratoomchat, B., Piyatiratitivorakul, S., Menasveta, P., 1993. Sperm quality of pond-reared and wild-caught *Penaeus monodon* in Thailand. J. World Aquacult. Soc. 24, 530 – 540.

Primavera, J. H., 1985. A review of maturation and reproduction in closed thelycum penaeids. In: Taki, Y., Primavera, J. H., Llobrera, J. A. (Eds.), Proc. 1st Int. Conf. on the Culture of Penaeid Prawns/ Shrimps. Aquaculture Department, SEAFDEC, Iloilo, Philippines, pp. 47 – 64.

Quackenbush, L. S., 1986. Crustacean endocrinology, a review. Can. J. Fish. Aquat. Sci. 43, 2271 – 2282.

Que-Tae, J., Laufer, H., Biggers, W., Sung, H., 1999. Methyl farnesoate induced ovarian maturation in the spider crab, *Libinia emarginata*. Invertebr. Reprod. Dev. 36, 79 – 85.

Shigekawa, K., Clark, W. H. Jr., 1986. Spermiogenesis in the marine shrimp, *Sicyonia ingentis*. Develop. Growth and Differ. 28, 95 – 112.

Sorgeloos, P., Lavens, P., Léger, P., Tackaert, W., Versichele, D., 1986. Manual for the culture and use of brine shrimp *Artemia* in aquaculture. State University of Ghent, Belgium, 319 pp.

Tabash, F. B., Palacios, J. V., 1996. Stock assessment of two penaeid prawn species, *Penaeus occidentalis* and *Penaeus stylirostris* (Decapoda: Penaeidae), in Golfo de Nicoya, Costa Rica. Rev. Biol. Trop. 44 (2), 595 – 602.

Wang, Q., Misamore, M., Jiang, C. Q., Browdy, C. L., 1995. Egg water induced reaction and biostain assay of sperm from marine shrimp *Penaeus vannamei*: dietary effects on sperm quality. J. World Aquacult. Soc. 26 (3), 261 – 271.

Wouters, R., Gomez, L., Lavens, P., Calderon, J., 1999. Feeding enriched *Artemia* biomass to *Penaeus vannamei* broodstock: its effect on reproductive performance and larval quality. J. Shellfish Res. 18 (2), 651 – 656.

Yashiro, R., Na-anant, P., Dumchum, V., 1998. Effect of methyltestosterone and 17 alpha-hydroxyprogesterone on spermatogenesis in the black tiger shrimp, *Penaeus monodon* Fab. In: Flegel, T. W. (Ed.), Advances in shrimp

biotechnology, National Center for Genetic Engineering and Biotechnology, Bangkok.

Summary

Reproduction of penaeid shrimp is currently practiced at commercial level all over the world, and the present state of the art allows a predictable production of fertilized eggs following a standard protocol. The major constraint for the advance of controlled reproduction is a lack of fundamental knowledge on the reproductive biology of both male and female shrimp. This thesis explores several aspects of shrimp reproduction, and evaluates practical alternatives for improving reproductive performance under controlled conditions.

As a start, the natural reproduction of the shrimp *Penaeus occidentalis* was studied between June 26 and July 27, 1992 near Curazao beach, Chira Island, Gulf of Nicoya, Costa Rica (**Chapter 2**). This is the first documentation of reproduction of a penaeid shrimp in the inner Gulf of Nicoya since the previous believe was that white shrimp reproduce only in deeper and oceanic waters. Spawning behavior was divided in five stages based on these observations; a sperm-egg interaction was studied using the *in vitro* spawning technique. Spawned eggs presented a cortical reaction during the first 30 min after release, and the sperm experienced primary binding to the egg vitelline envelope, but an acrosome reaction was not observed. Wild *P. occidentalis* males presented heavy spermatophores compared to other open thelycum shrimps. Sperm count (49.52 million per compound spermatophore), and sperm abnormalities (22.0 %), were similar to those of wild *P. setiferus* from the Gulf of Mexico.

The preliminary results obtained with *in vitro* spawning of wild *P. occidentalis* suggest that *in vitro* fertilization and induction of the acrosome reaction of open thelycum shrimp require more research before practical *in vitro* fertilization techniques for science and industry can be defined.

Knowledge about male maturation and semen quality is fragmentary, with little information available concerning the way a male produces spermatophores. Captive shrimp often present fertility problems, the most important one being blackening of the male reproductive system, which is a deleterious condition, and which has been observed in different penaeid species kept in captivity. *P. setiferus* is particularly susceptible to this condition. The genital apparatus of male penaeid shrimp, *P. setiferus*, blackens with resulting detrimental effects on mating for production of larvae when animals are kept in controlled maturation/reproduction situations. A progressive, melanized condition of the male reproductive tract was shown to be associated with bacterial infection (**Chapter 3**). At least three different species (*Vibrio alginolyticus*, *Pseudomonas putrefaciens*, and an unclassified strain), were isolated from damaged tissues and successfully developed the same signs in challenge experiments.

The deterioration of spermatophores in captive *P. setiferus* is a predictable event. The process involves not only the spermatophores but also the entire reproductive tract producing sterile males in a short period of time (1 mo). It is suggested that the condition could be a progressive syndrome with bacterial invasion perhaps only in the advanced stages, or that more than one etiology may be involved in deterioration and blackening of *P. setiferus* reproductive system. Bacterial infection is not the primary cause of this problem as no evidence was found of bacterial infection being associated with the onset of the condition. Stress was suggested as a possible first cause of the syndrome.

Results from chapter 3 indicated that more observations on the onset of spermatophore deterioration in other penaeid shrimp were required. Quality and deterioration of spermatophores have been observed in the commercially important pond-grown *P. stylirostris* and *P. vannamei;* these species were therefore selected for a second and third study.

Several male *P. stylirostris* were selected from a 3-ha commercial earthen pond and were individually evaluated for reproductive performance (**Chapter 4**). Indicators measured were compound spermatophore weight, sperm count, and sperm abnormalities. It was found that spermatophore quality was significantly better for 30-40 g shrimp than for 20-30 g shrimp ($P < 0.05$). The higher frequency of abnormalities measured in younger males and the inverse relationship between abnormalities and sperm count indicate that the vas deferens could be the tissue responsible for producing highly abnormal immature semen. We propose that male maturation has at least three independently controlled levels of organization: testes maturation, vas deferens maturation, and spermatophore synthesis. The individual evaluation showed that each male followed a particular response in reproductive quality. Changes in spermatophore weight were not an indicator of sperm density within spermatophores. Male reproductive tract degenerative syndrome (MRTDS) and male reproductive system melanization (MRSM) did not develop in any shrimp during our experiments.

In **Chapter 5** the effects of male eyestalk ablation and laboratory culture on spermatophore quality and spermatophore deterioration in young pond-grown *P. vannamei* were evaluated. Sperm production of males collected from grow-out ponds was increased by two techniques: eyestalk ablation in combination with a fresh frozen maturation diet (13% body weight); and laboratory culture using a diet of the formulated feed Nicovita Plus (3% body weight/ d) and frozen squid (2%). Findings are complementary to previous reports that eyestalk ablation improves quality of spermatophores in young (25.7 g) males. The timing of eyestalk ablation for activation of the endocrine mechanism, leading to improved spermatophore quality was also

explored. After eyestalk ablation, performed between postmolt and intermolt stages, 26 g males required a minimum of three spermatophore regenerations or 42 d to significantly increase spermatophore size and sperm count. On the other hand, the laboratory culture (2.5 mo) technique improved the quality of spermatophores in successive regenerations for non-ablated males. In the present study, subadult *P. vannamei* produced spermatophores which, if not transferred or manually ejaculated, gradually deteriorated (successive stages are described), while a new compound spermatophore was being synthesized.

In the previous chapters it was demonstrated that spermatophore quality can be improved by eyestalk ablation and laboratory culture in young *P. vannamei.* In **Chapter 6** the effects of a single injection of 17-alpha-methyltestosterone and 17-alpha-hydroxyprogesterone on sperm quality was investigated. These molecules were chosen because they have been identified from androgenic gland extracts, and were suggested to play complementary roles to the androgenic gland hormone. Spermatophores of white shrimp, *P. vannamei* were improved, in terms of increased sperm count, spermatophore weight, and a reduced incidence of sperm abnormalities by a single injection of 17-alpha-methyltestosterone at 0.01 or 0.1 $\mu g\ g^{-1}$ body weight (b.w.). 17-alpha-hydroxyprogesterone did not induce a significant improvement in spermatophore quality. These findings indicate that a steroid injection program can be a practical alternative for improving spermatophore quality in commercial operations.

Unilateral eyestalk ablation is still the major technique for predictable induction of ovarian maturation and spawning in penaeid shrimp. However, predictable induced reproduction in captive penaeids without the use of eyestalk ablation is considered to be the long term goal for shrimp mariculture. In **Chapter 7** the effect of serotonin (5-hydroxytriptamine, 5-HT) injection on the ovarian maturation and spawning of wild *P. vannamei* was investigated. The neurotransmitter was evaluated at 15 $\mu g\ g^{-1}$ b.w. and 50 $\mu g\ g^{-1}$ b.w., applied at day 1, 11 and 21. The effect was compared against a control group, which received the injection of the sterile vehicle solution (NaCl 0.85%), and an unilaterally eyestalk ablated group. 5-HT induced ovarian maturation and spawning at both doses tested, generating more spawnings at 50 $\mu g\ g^{-1}$ b.w.; however, unilateral eyestalk ablation induced a sooner and a higher rate of maturation and spawning. Our findings may be the result of 5-HT interaction with the release of different neurohormones and inhibition of methyl farnesoate synthesis. Additionally, gonad inhibiting hormone may have an intense control over ovaries and hepatopancreas. Spawnings obtained by serotonin treatment showed excellent quality, and it was not statistically different ($P > 0.05$) from unilaterally eyestalk ablated females. This study

demonstrated that serotonin induced ovarian maturation and good quality spawning. The results obtained with serotonin injections have been confirmed by more recent reports about endocrine control of reproduction in decapod crustaceans; however, based on the present understanding of shrimp endocrinology, a "combined molecular approach", i.e. injecting different hormones, should be explored.

In the last chapter of this thesis (**Chapter 8**), the effects of cooling and cryoprotectants on shrimp embryo survival were studied as a first step for developing a cryogenic protocol. Cryopreservation of penaeid shrimp seedstock would make international trading more economical and efficient, and it has been suggested as an important advance for future breeding programs.

The sensitivity of embryos of the penaeid shrimp, *Trachypenaeus byrdi*, to cooling, cryoprotectant exposure (dimethyl sulfoxide: DMSO, sucrose, methanol and glycerol), and hypersaline treatment, was assessed. In addition, cooling and DMSO exposure was evaluated in *P. stylirostris* and *T. byrdi* nauplii. Morulae and advanced embryos (setae development stage) showed tolerance to cooling at 10 °C, but were very sensitive to 0 °C exposure. Methanol exposure at 12 °C up to 2 M, was non toxic for advanced embryos. DMSO toxicity was intermediate; no statistical decrease in survival ($P > 0.05$) was measured at 0.5 M. Sucrose and glycerol were toxic to both embryo stages over 0.25 M and 0.5 M, respectively. Morulae were more resistant to hypersaline treatment at 55 ppt than advanced embryos. Nauplii showed a better tolerance to cooling and DMSO exposure than embryos. These findings are being applied to develop a cryogenic protocol for penaeid embryos.

Shrimp embryos showed great tolerance to cooling, and this characteristic is suggested to be related to the presence of omega-3 PUFAs in penaeid eggs. It was also found that the cooling tolerance increases with the developmental stage, with the nauplius larvae being the most resistant. Ethylene glycol and methanol are the most suitable candidates, so far tested, for developing cryogenic protocols with shrimp embryos. Hypersaline dehydration does not seem adequate for shrimp embryos since they proved highly sensitive to increased salt concentration. It can be concluded that while the efforts on cryopreservation of shrimp larvae have been largely unsuccessful, embryos may prove to be a promising alternative for cryopreservation.

Samenvatting

Voortplanting van garnalen voor commerciele doeleinden vindt momenteel over de gehele wereld plaats, waarbij, gebruikmakend van eenvoudige standaard protocollen, een redelijk betrouwbare productie mogelijk is. De belangrijkste beperking bij de verdere ontwikkeling van technieken voor gecontroleerde voortplanting is het ontbreken van fundamentele kennis over de reproductie biologie van mannelijke en vrouwelijke garnalen. In deze thesis worden diverse aspecten van de voortplanting van garnalen nader bestudeerd, en praktische alternatieven voor de verbetering van de voortplantings capaciteit geevalueerd.

Eerst werd de natuurlijke voortplanting van de garnaal *Penaeus occidentalis* bestudeerd in de periode van 26 juni en 27 juli, 1992, nabij playa Curazao, Isla Chira, in de golf van Nicoya in Costa Rica (**Hoofdstuk 2**). Dit is de eerste beschrijving van de natuurlijke voortplanting van Penaeidae garnalen in het ondiepere binnenste deel van de golf, aangezien de algemene opvatting was dat garnalen zich alleen in de diepere gedeelten van de golf en in de oceaan voortplanten. De voortplanting werd verdeeld in een vijftal stadia. De interactie tussen sperma en ei tijdens de bevruchting werd bestudeerd met behulp van een *in vitro* fertilizatie techniek. Geovuleerde eieren ondergingen een corticale reactie gedurende de eerste 30 minuten na vrijkomen, waarbij het sperma zich aan de vitelline membraan van het ei hechtte. Een acrosoom reactie werd echter niet waargenomen. Wilde *P. occidentalis* mannetjes hadden zware spermatoforen in vergelijking met andere zgn. "open thelycum" garnalen species. Sperma hoeveelheden (49.52 miljoen per spermatofoor) en sperma afwijkingen (22.0 %) waren vergelijkbaar met die van wilde *P. setiferus* uit de golf van Mexico.

De voorlopige resultaten verkregen met de in vitro fertilizatie techniek van wilde *P. occidentalis* tonen aan dat meer onderzoek nodig is naar "*in vitro*" fertilizatie en acrosoom inductie bij open thelycum garnalen voordat praktische protocollen voor *in vitro* fertilizatie voor de industrie en wetenschap kunnen worden ontwikkeld.

Kennis over de mannelijke voortplanting en sperma kwaliteit is fragmentarisch, en weing kennis is beschikbaar over de manier waarop garnalen spermatoforen produceren. Garnalen in gevangenschap hebben vaak vruchtbaarheids problemen, waarvan de belangrijkste het zwart worden van het mannelijke voortplantings orgaan is. Dit is een lethale conditie die bij diverse penaeide soorten in gevangenschap is waargenomen. *P. setiferus* is vooral gevoelig voor deze aandoening. Het genitale stelsel van mannelijke *P. setiferus* wordt zwart met nadelige gevolgen voor de voortplanting en de productie van larven wanneer de dieren onder gecontroleerde voortplantings condities worden gehouden. Deze voortschrijdende "melanizatie" van het mannelijke

genitale stelsel is geassocieerd met een bacteriele infectie (**Hoofdstuk 3**). Minstens drie soorten bacterien (*Vibrio alginolyticus, Pseudomonas putrefaciens* en een onbekende soort) werden geisoleerd uit aangetast weefsel, en induceerden vervolgens dezelfde symptomen in challenge experimenten. Het afsterven van spermatoforen in *P.setiferus*, gehouden in gevangenschap is een voorspelbare gebeurtenis. Het proces heeft niet alleen betrekking op de spermatoforen, maar op het gehele genitale stelsel, waardoor binnen 1 maand steriliteit optreed. Het is mogelijk dat er sprake is van een progressief syndroom waarbij de bateriele infectie alleen optreed in de meer vergevorderde stadia, of dat er sprake is van meer dan een etiologie bij het afsterven van het genitale apparaat. Bacteriele infectie is zeker niet de primaire oorzaak van dit probleem aangezien er geen bewijs werd gevonden voor een bacteriele infectie in de vroege stadia van de aandoening. Waarschijnlijk is stress de eerste oorzaak voor het optreden van het syndroom.

De resultaten van hoodstuk 3 gaven aanleiding tot verdere bestudering van de degeneratie van spermatoforen in andere species. Aangezien vermindering van kwaliteit en afsterven van spermatoforen ook bij de commercieel belangrijke *P. vannamei* en *P. stylirostris* optreed, werden deze species gebruikt voor een tweede (hoofdstuk 4) en derde (hoofdstuk 5) studie.

Verschilende mannelijke *P. stylirostris* werden geselecteerd uit een 3 ha grote commerciele aarden vijver en individueel beoordeeld op voortplantings kenmerken (**Hoofdstuk 4**). De kenmerken waren: totaal gewicht van de spermatoforen, aantal spermatozoiden per spermatofoor, en sperma afwijkingen. Uit deze studie bleek dat de sperma kwaliteit van 30-40 g dieren significant beter was in vergelijking met kleinere (20-30 g) garnalen. De hogere frequentie van sperma afwijkingen in jongere mannetjes en de inverse relatie tussen sperma afwijkingen en sperma aantallen gaven aan dat de vas deferens mogelijk betrokken was bij de produktie van grote aantallen abnormaal immatuur sperma. Mannelijke maturatie kent drie onafhankelijk van elkaar gecontroleerde niveaus van organizatie, t.w. testis maturatie, vas deferens ontwikkeling en spermatofoor synthese. Veranderingen in spermatofoor gewicht waren niet gerelateerd aan de hoeveelheid spermatozoiden in een spermatofoor. Het "male reproductive tract degenerative syndrome" (MRTDS) en "male reproductive system melanization" (MRSM) werd in geen van de dieren waargenomen.

In **Hoofdstuk 5** werden de effecten van oogsteel amputatie en laboratorium kweek op de spermakwaliteit en spermatofoor degeneratie in jonge *P. vannamei*, afkomstig van vijvers, bestudeerd.

De spermaproductie van mannetjes afkomstig uit opkweek vijvers werd vergroot door twee technieken, t.w. oogsteel amputatie in combinatie met het voeren

van een versgevroren maturatie dieet, en laboratorium opkweek op een dieet van Nicovita plus (3% lichaamsgewicht/dag) en bevroren inktvis (2 % lichaamsgewicht /dag). De bevindingen zijn complementair aan eerdere publikaties, waaruit blijkt dat oogsteel amputatie de kwaliteit van spermatoforen in jonge mannetjes (gemidd. 25.7 g) verbeterd.

Het tijdstip waarop oogsteel amputatie moet plaatsvinden om de endocrine activatie te bewerkstelligen welke leidt tot verbeterde spermatofoor kwaliteit werd eveneens onderzocht. Na oogsteel amputatie, uitgevoerd tussen post-vervelling en vervellen, hadden jonge mannetjes (26 g) gemiddeld minimaal 3 spermatofoor regeneraties, corresponderend met 42 dagen, nodig voordat er sprake was van een significante toename in spermatofoor gewicht en sperma aantallen. In laboratorium kweek (2.5 mnd) van niet geamputeerde mannetjes daarentegen verbeterde de kwaliteit van spermatoforen met opeenvolgende regeneraties. In deze studie produceerde sub adulte *P. vannamei* spermatoforen welke, indien niet manueel geejaculeerd, geleidelijk aan degenereerden terwijl gelijktijdig een nieuwe spermatofoor werd gesynthetiseerd.

In het vorige hoofdstuk werd aangetoond dat de spermatofoor kwaliteit van jonge *P. vannamei* verbeterd kan worden door oogsteel amputatie en laboratorium kweek. In **Hoofdstuk 6** werden de effecten van een enkelvoudige injectie van 17 alpha methyltestosteron en 17 alpha hydroxyprogesteron op de sperma kwaliteit bestudeerd. Deze steroiden waren gekozen omdat ze voorkomen in extracten van de androgene klier, en omdat wordt aangenomen dat ze een complementaire rol spelen bij het androgene klier hormoon.

Spermatoforen van *P. vannamei* waren significant verbeterd in termen van spermatofoor gewicht, aantallen spermatozoiden en % abnormaal sperma, na een enkele injectie van 0.1 of 0.01 µg 17 alpha methyltestosteron per gram lichaamsgewicht. Injecties met 17 alpha hydroxyprogesteron gaven geen significante verbetering van spermatofoor kwaliteit. Deze bevindingen laten zien dat steroiden therapie een praktisch alternatief kan zijn om de spermatofoor kwaliteit in garnalen te verbeteren.

Eenzijdige oogsteel amputatie is nog steeds de meest gebruikte techniek om betrouwbaar maturatie en ovulatie in vrouwelijke penaeide garnalen te induceren. Op de langere termijn dient deze techniek echter vervangen te worden door betere, niet invasieve, hormonaal geinduceerde voortplantings technieken.

In **Hoofdstuk 7** werd het effect van serotonine (5-hydroxytriptamine, 5-HT) op de ovariele maturatie en ovulatie van wilde *P. vannamei* bestudeerd. Twee doses werden getest, tw. 15 en 50 µg per gram lichaamsgewicht, toegediend op dag 1, 11 en

21 van het experiment. De effecten werden vergeleken met een blanco geinjecteerde controle groep (0.85% NaCl) en een groep dieren welke eenzijdige oogsteel amputatie hadden ondergaan. 5-HT induceerde ovariele maturatie en ovulatie in beide geteste doses, terwijl de meeste ovulaties werden waargenomen na injectie met 50 µg 5-HT/gram lichaamsgewicht.

Eenzijdige oogsteel amputatie induceerde echter een significant snellere response en een hoger percentage maturatie en ovulatie dan 5-HT. Deze bevindingen kunnen het resultaat zijn van een interactie van 5-HT met de afgifte van andere neuropeptiden en remming van methyl farnesoate synthese. Het is ook mogelijk dat het "gonad inhibiting hormone" (GIH) in de oogsteel een volledige en stringente controle uitoefent op het ovarium en de hepatopancreas. Voortplantingen na serotonine behandeling waren uitstekend van kwaliteit en niet significant verschillend van voortplantingen verkregen na oogsteel amputatie. Deze studie toonde aan dat behandeling met sertonine een goede maturatie en ovulatie kan induceren, en tot een goede kwaliteit voortplantingen kan leiden. Recent zijn deze bevindingen bevestigd door andere studies naar de endocrine regulatie van de voortplanting bij garnalen. Uitgaande van deze bevindingen verdient het aanbeveling om een gecombineerde moleculaire benadering te testen, dwz combinaties van meerdere hormonen te injecteren.

In het laatste hoofdstuk van deze thesis (**Hoofdstuk 8**) werd het effect van afkoeling en cryoprotectanten op garnalen embryo overleving bekeken, als zijnde een eerste stap naar het ontwikkelen van een protocol voor cryoconservering. Cryoconservering van Penaeidae embryos wordt gezien als een belangrijke stap in het verbeteren en uitbreiden van de internationale handel in garnalen broed en een zeer belangrijke stap in het ontwikkelen van toekomstige fokprogrammas.

De gevoeligheid van embryos van de Penaeidae garnaal *Trachypenaeus byrdii* voor afkoelen, blootstelling aan cryoprotectanten (DMSO, sucrose, methanol en glycerol) en hypersaliniteit werd bepaald. Daarnaast werden de gevolgen van afkoeling en blootstelling aan DMSO ook getest in *P. styliostris* and *T. byrdii* nauplii. Morulae en meer ontwikkelde embryos (setae ontwikkelings stadium) tolereerden afkoeling tot 10 C , maar waren erg gevoelig voor afkoeling naar 0 C Methanol blootstelling bij 12 C , tot 2 M, was niet toxisch voor embryos in het setae stadium. DMSO was niet toxisch bij concentraties tot 0.5 M. Sucrose en glycerol waren toxisch voor morula en setae embryos bij concentraties hoger dan 0.25 en 0.5 M respectievelijk. Morula waren meer bestand tegen hypersaliniteit (55 ppt) dan setae embryos. Nauplii tenslotte tolereerden afkoeling en DMSO blootstelling beter dan embryos. Deze bevindingen kunnen worden

gebruikt voor de ontwikkeling van een cryoconserverings protocol voor Penaeidae embryos.

Garnalen embryos hebben een redelijke grote tolerantie voor afkoeling, iets wat in verband gebracht kan worden met de aanwezigheid van grote hoeveelheden omega-3 PUFA's in Penaeidae embryos. De tolerantie voor afkoeling nam bovendien toe met de ontwikkeling van de embryos, waabij nauplii de grootste tolerantie vertoonden. Ethyleen glycol en methanol zijn de meest geschikte kandidaten, tot dusver getest, voor de ontwikkeling van cryoconserverings protocollen. Hypersaline dehydratie lijkt niet geschikt voor Penaeidae garnalen gegeven de grote gevoeligheid voor verhoogde zoutconcentraties. Concluderend kan gesteld worden dat pogingen voor cryoconservering van garnalen larven tot nu toe zonder succes zijn geweest, maar dat embryos een veelbelovend alternatief voor cryoconservering kunnen zijn.

Resumen

La reproducción de camarones peneidos se practica a nivel comercial en todo el planeta, y el estado actual del conocimiento permite alcanzar producciones predecibles de huevos fértiles, siguiendo un protocolo establecido. La mayor restricción para el avance de la reproducción controlada es la carencia de conocimiento fundamental en la biología reproductiva de machos y hembras. Esta tesis explora varios aspectos de la reproducción de camarones, y evalúa alternativas prácticas para el mejoramiento de la calidad reproductiva en condiciones controladas.

Para comenzar, se estudió la reproducción natural del camarón, *Penaeus occidentalis*, entre Junio 26 y Julio 27, 1992, cerca de Playa Curazao, Isla Chira, Golfo de Nicoya, Costa Rica (**Capítulo 2**). Este es el primer reporte de reproducción de un camarón peneido en la zona interna del Golfo de Nicoya, ya que la creencia anterior era que los camarones blancos se reproducían solamente en aguas más profundas y oceánicas. Según nuestras observaciones, el comportamiento de desove se dividió en cinco estados. Se estudió la interacción esperma-huevo usando la técnica de desove *in vitro*. Los huevos desovados experimentaron una reacción cortical durante los primeros 30 min después de la liberación, y los espermatozoides experimentaron unión primaria con la envoltura vitelina de los huevos, pero no se observó reacción acrosomal. Los machos silvestres de *P. occidentalis* mostraron espermatóforos grandes, en comparación con otros camarones de télico abierto. El conteo de espermatozoides (49,52 millones por espermatóforo compuesto) y las anormalidades de espermatozoides (22,0%) fueron similares a los valores medidos en *P. setiferus* silvestres del Golfo de México.

Los resultados preliminares obtenidos con el desove *in vitro* de *P. occidentalis* silvestres sugieren que la fertilización *in vitro* y la inducción de la reacción acrosomal de camarones de télico abierto, requieren más investigación antes de poder ofrecer a la ciencia y la industria, técnicas prácticas de fertilización *in vitro*.

El conocimiento sobre maduración de machos y calidad del semen es incompleto. Los camarones en cautiverio a menudo presentan problemas de fertilidad, siendo el más importante el ennegrecimiento del sistema reproductor masculino, observado en diferentes especies de peneidos mantenidos en cautiverio. *P. setiferus* es particularmente susceptible a esta condición. El aparato genital de machos de *P. setiferus* se torna ennegrecido, con efectos dañinos para el apareamiento y la producción de larvas. Cuando los animales se mantienen en condiciones de maduración y reproducción, una condición de melanización progresiva del tracto reproductor masculino demostró estar asociada con infección bacteriana (**Capítulo 3**). Al menos

tres diferentes especies (*Vibrio alginolyticus, Pseudomonas putrefaciens*, y una cepa no identificada) fueron aisladas de tejidos dañados y desarrollaron los mismos signos en experimentos de infectividad.

El deterioro de espermatóforos en *P. setiferus* cautivos es un evento predecible. El proceso involucra los espermatóforos y el tracto reproductivo completo, produciendo machos estériles en un corto tiempo (1 mes). Se sugiere que la condición podría ser un síndrome progresivo con invasión bacteriana, quizás solamente en los estados avanzados, o que más de una etiología puede estar involucrada en la deterioración y el ennegrecimiento del sistema reproductor de *P. setiferus*. Infección por bacterias no es la causa primaria de este problema ya que no se encontraron evidencias de infección asociada con la manifestación de esta condición. El estrés fue sugerido como una posible causa del síndrome.

Los resultados del capítulo 3 indican que se necesitan más observaciones sobre deterioración de espermatóforos en otros camarones peneidos. La calidad y deterioración de espermatóforos se han observado en camarones de importancia comercial (*P. stylirostris* y *P. vannamei)*, crecidos en estanques. Por lo tanto, estas especies se seleccionaron para un segundo y tercer estudio.

Machos de *P. stylirostris* se seleccionaron de un estanque de tierra comercial de 3-ha, y fueron evaluados individualmente en cuanto a su potencial reproductivo (**Capítulo 4**). Los indicadores medidos fueron: peso del espermatóforo compuesto, recuento de espermatozoides, y anormalidad de espermatozoides. Se encontró que la calidad de los espermatóforos fue significativamente mejor en camarones de 30 – 40 g, en comparación con camarones de 20 – 30 g ($P < 0,05$). La mayor frecuencia de anormalidades medidas en los camarones más jóvenes, y la relación inversa entre anormalidades y recuento de espermatozoides, indican que el vaso deferente podría ser el tejido responsable de la producción de semen inmaduro con altas anormalidades. Se propone que la maduración de machos tiene al menos tres niveles de organización con control independiente: maduración en testículos, maduración en los vasos deferentes, y síntesis de espermatóforos. La evaluación individual mostró que cada macho sigue una respuesta particular en calidad reproductiva. Los cambios en el peso de los espermatóforos no fueron un indicador de la densidad de espermatozoides dentro de los mismos. El síndrome degenerativo del tracto reproductor masculino (SDTRM), y la melanización del sistema reproductor masculino (MSRM) no se desarrollaron en ningún camarón durante los experimentos.

En el **Capítulo 5** se evaluaron los efectos de la ablación ocular de machos y el cultivo en laboratorio, sobre la calidad y deterioración de espermatóforos en *P. vannamei jó*venes, crecidos en estanques. La producción de espermatozoides de

machos colectados en estanques de engorde se mejoró mediante dos técnicas: ablación ocular en combinación con una dieta fresca-congelada de maduración (13% peso corporal), y cultivo en laboratorio usando una dieta formulada de Nicovita Plus (3% peso corporal/ día) y calamar congelado (2%). Los resultados son complementarios a reportes previos, ya que la ablación ocular mejoró la calidad de los espermatóforos en machos jóvenes de 25,7 g de peso. Se evaluó el tiempo requerido, luego de la ablación ocular, para activar el mecanismo endocrinológico que conlleva al mejoramiento de la calidad de los espermatóforos. Después de la ablación ocular, practicada entre los estados de post-muda e inter-muda, machos de 26 g necesitaron un mínimo de tres regeneraciones de espermatóforos (42 días) para mejorar significativamente el tamaño del espermatóforo y el recuento de espermatozoides. Por otro lado, la técnica de cultivo en laboratorio (2,5 meses) mejoró la calidad de los espermatóforos en regeneraciones sucesivas en machos no ablacionados. En este estudio, subadultos de *P. vannamei* produjeron espermatóforos que, de no ser transferidos o eyaculados manualmente, se deterioraban gradualmente (los estados sucesivos son descritos), a la vez que un nuevo espermatóforo compuesto se estaba sintetizando.

En los capítulos anteriores se demostró que la calidad de los espermatóforos puede ser mejorada mediante ablación ocular y cultivo en laboratorio de *P. vannamei* jóvenes. En el **Capítulo 6** se investigó los efectos de una sola inyección de 17-alfa-metiltestosterona y 17-alfa-hidroxiprogesterona, sobre la calidad del semen. Estas moléculas se escogieron porque han sido identificadas en extractos de glándula androgénica, y se ha sugerido que juegan un papel complementario a la hormona de la glándula androgénica. Mediante una sola inyección de 17-alfa-metiltestosterona a razón de 0,01 y 0,1 $\mu g\ g^{-1}$ peso corporal (p.c.), los espermatóforos del camarón blanco, *P. vannamei*, fueron mejorados en términos de un mayor recuento de espermatozoides y peso del espermatóforo, y una menor incidencia de espermatozoides anormales. Por su parte, la 17-alfa-hidroxiprogesterona no indujo ningún mejoramiento significativo en la calidad de los espermatóforos. Estos resultados indican que un programa de inyección de esteroides puede ser una alternativa práctica para mejorar la calidad de espermatóforos en operaciones comerciales.

La ablación ocular unilateral es aún la mejor técnica para inducir de forma predecible la maduración ovárica y el desove en camarones peneidos. Sin embargo, la inducción de la reproducción en peneidos cautivos, sin el uso de la ablación ocular, se considera como un objetivo a largo plazo en maricultura de camarones. En el **Capítulo 7** se evaluó el efecto de la inyección de serotonina (5-hidroxitriptamina, 5-HT) sobre la maduración ovárica y el desove de *P. vannamei* silvestres. El neurotransmisor se evaluó

a razón de 15 μg g^{-1} p.c. y 50 μg g^{-1} p.c., aplicado los días 1, 11, y 21. El efecto se comparó contra un grupo control que recibió la inyección de la solución vehículo estéril (NaCl 0.85%), y con un grupo de hembras con pedúnculos oculares ablacionados unilateralmente. 5-HT indujo la maduración ovárica y el desove en ambas dosis, generando más desoves con 50 μg g^{-1} p.c. Sin embargo, la ablación unilateral del pedúnculo ocular indujo una más pronta y mayor tasa de maduración y desove. Estos resultados pueden ser producto de la interacción entre 5-HT con la liberación de diferentes neurohormonas y la inhibición de la síntesis de metil farnesoato. Adicionalmente, la hormona inhibitoria de la gónada puede tener un control intenso sobre los ovarios y el hepatopáncreas. Los desoves obtenidos mediante tratamiento con serotonina mostraron excelente calidad, y no fueron estadísticamente diferentes ($P > 0,05$) de las hembras con pedúnculos oculares ablacionados. Este estudio demostró que la serotonina induce la maduración ovárica y la buena calidad de los desoves. Los resultados obtenidos con inyecciones de serotonina han sido confirmados por recientes reportes sobre el control endocrino de la reproducción en crustáceos decápodos. Sin embargo, con base en el conocimiento actual de la endocrinología de camarones, se debería explorar un "enfoque molecular combinado", por ejemplo, inyectando diferentes hormonas.

En el último capítulo de esta tesis (**Capítulo 8**), se estudió el efecto del enfriamiento y crioprotectores sobre la sobrevivencia de embriones, como un primer paso en el desarrollo de un protocolo criogénico. La criopreservación de larvas de camarones peneidos permitiría un comercio internacional más eficiente, y sería un avance importante para programas de apareamiento futuros.

Se estudió la sensibilidad de los embriones del camarón peneido, *Trachypenaeus byrdi*, al enfriamiento, a la exposición a diversos crioprotectores (dimetil sulfóxido: DMSO, sucrosa, metanol, y glicerol), y al tratamiento hipersalino. Además, se evaluó el enfriamiento y la exposición al DMSO en nauplios de *P. stylirostris* y *T. byrdi*. Las mórulas y los embriones avanzados (estado de desarrollo de setas) mostraron tolerancia al enfriamiento a 10 °C, pero fueron muy sensibles a la exposición a 0 °C. La exposición al metanol a 12 °C hasta 2 M, no fue tóxica para los embriones avanzados. La toxicidad al DMSO fue intermedia; no se registró bajas estadísticas ($P > 0,05$) en la sobrevivencia a 0,5 M. La sucrosa y el glicerol fueron tóxicos para ambos estados embrionarios a concentraciones superiores a 0,25 M y 0,5 M, respectivamente. Los embriones en estado de mórula fueron más resistentes al tratamiento hipersalino (55 partes por mil) que los embriones avanzados. Los nauplios mostraron una mejor tolerancia al enfriamiento y a la exposición al DMSO, que los

embriones. Estos resultados están siendo aplicados para desarrollar un protocolo criogénico para embriones de peneidos.

Los embriones de camarones mostraron una gran tolerancia al enfriamiento, y se sugiere que esta característica está relacionada con la presencia de ácidos grasos poliinsaturados omega 3, en huevos de peneidos. También se encontró que la tolerancia al enfriamiento aumenta con el estado de desarrollo, siendo la larva nauplio la más resistente. El etilenglicol y el metanol son los mejores medios, probados hasta el momento, para desarrollar protocolos criogénicos con embriones de camarones. La deshidratación hipersalina no parece ser adecuada para embriones de camarones ya que éstos demostraron ser altamente sensibles al aumento de la concentración salina. Se concluye que mientras los esfuerzos en criopreservación de larvas de camarones no han sido exitosos, la criopreservación de los embriones puede resultar ser una alternativa prometedora.

Acknowledgements

The articles upon which this thesis was elaborated were published in cooperation with other people. I thank my colleagues, José Palacios, Ramón Angulo, and Tito Aldave. The participation of my graduate students, Xenia Lozano and Alí Vaca, was crucial. I also thank my professors from Texas A&M University, Addison Lawrence and Don Lewis.

I feel deeply grateful to Johan Verreth and Marc Verdegem for their interest and support to make this project possible. To my promoter, Bram Huisman, and my co-promoter, Hans Komen, for the opportunity, the guidance, and the trust for completing my PhD.

Special thanks to my friend, Ricardo Jiménez, for his permanent advice during the writing of this thesis. To Escuela de Ciencias Biológicas, Universidad Nacional, Costa Rica, for its support during all these years of research.

To Anne van Dam and Beatriz Torres for their assistance during the defense and graduation ceremony. To my girlfriend, Ivannia Vargas, for her kind love and friendship.

Curriculum vitae

Jorge Alfaro Montoya was born on the 5th of March, 1962, in Alajuela, Costa Rica. He obtained his BSc degree in Marine Biology in 1982 at the Universidad Nacional. In 1985, he obtained a postgraduate degree in Marine Biology, with emphasis on Aquaculture, from the same university. After his graduation, he was employed by Escuela de Ciencias Biológicas, Universidad Nacional, Costa Rica as a lecturer and a researcher. In 1987, he obtained a scholarship from the U.S. International Agency for Cooperation, and enrolled as a MSc student in the Wildlife and Fisheries Department of Texas A&M University. In 1990, he obtained his MSc in Aquaculture. Since 1990 he has been teaching courses in aquaculture and doing research in the field of shrimp reproduction at the Universidad Nacional, C.R. He has published 17 scientific papers on oyster and shrimp mariculture.

Controlled reproduction of penaeid shrimp: a contribution to its improvement

Alfaro Montoya, Jorge

Doctoral dissertation, Fish Culture and Fisheries Group, Wageningen Institute of Animal Sciences (WIAS), Wageningen University, P.O. Box 338, 6700 AH, Wageningen, The Netherlands.

ISBN: 90-5808-400-0
Cover design: Jorge Alfaro Montoya and Valeria Alfaro Vargas
Printing: Ponsen & Looijen, Wageningen

Abstract

This dissertation deals with controlled reproduction of penaeid shrimp. New knowledge about natural reproductive activity of *Penaeus occidentalis* in Gulf of Nicoya, Costa Rica, is presented. Since *in vitro* fertilization of open thelycum shrimp proved unsuccessful, a hypothesis is given to explain experimental results. In *P. setiferus*, the Male Reproductive Blackening Disease was studied, and bacterial infection was found to be associated with the male's condition. Production of spermatophores in captivity was explored in two species, *P. stylirostris* and *P. vannamei*. Adequate husbandry as well as successive ejaculation improved spermatophore quality. Deterioration of spermatophores was observed as part of a normal process for renewal in *P. vannamei*, without pathological implications. In order to further improve spermatophore quality, the injection of 17-alpha-methyltestosterone and 17-alpha-hydroxyprogesterone at 0.01 and 0.1 μg g^{-1} body weight was evaluated. 17-alpha-methyltestosterone significantly improved the quality of spermatophores, whereas 17-alpha-hydroxyprogesterone did not. Serotonin injection was evaluated as an alternative to female's eyestalk ablation for induction of ovarian maturation and spawning in *P. vannamei*. This neurotransmitter induced lower maturation and spawning with 3 doses of 50 μg g^{-1} body weight, than eyestalk ablation. In other to lay a basis for cryopreservation, penaeid embryos were evaluated in terms of their tolerance to cooling, cryoprotectants, and hypersaline solutions. *T. byrdi* morulae and advanced embryos (10 h) were tolerant to cooling at 10 °C, but were very sensitive to 0 °C. Embryos showed high tolerance to methanol and intermediate tolerance to dimethyl sulfoxide. Morulae were more resistant to hypersaline treatment at 55 ppt than advanced embryos.