BARD

FINAL REPORT

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**The Establishment of Monosex Populations
in the Freshwater Prawn
*Macrobrachium Rosenbergii***

D. Cohen, E.S. Chang

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Title

The Establishment of Monosex Populations in the Freshwater

Prawn Macrobrachium rosenbergii

Investigators' Names
(Principal listed first)

Dan Cohen

Ernest S. Chang

Investigators' Institutions

The Hebrew University of Jerusalem

University of California, Davis

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Signature
Principal Investigator

Signature
Institution's Authorizing Official

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ANNEX 2: Sagi, A., Milner, Y. and Cohen, D., 1988. Spermatogenesis and sperm storage in the testes of behaviorally distinctive male morphotypes of Macrobrachium rosenbergii (Decapoda, Palaemonidae). Biol. Bull., 174:330-336.

ANNEX 3: Sagi, A., Cohen, D. and Y. Milner, 1989. Effect of androgenic gland ablation on morphotypic differentiation and sexual characteristics of male freshwater prawns Macrobrachium rosenbergii. Gen. Comp. Endocrinol., in press.

ANNEX 4: Sagi, A., Karp, L., Milner, Y., Cohen, D., Kuris, A.M. and E. Chang. Variation in spermatogenesis during the molt cycle of Macrobrachium rosenbergii (Decapoda, Palaemonidae) - direct effect of 20-Hydroxyecdysone in vitro., submitted for publ.

1. Project Results which were already Published or are Currently in the Publication Process.

1.1 Establishment of in vivo methods for the evaluation of growth and reproduction in males.

In the course of this part of the study we aimed at using anatomical tools such as evaluation of the development of the reproductive system and midgut gland to be used as tested parameters in the in vivo bioassay of the induction of maleness in prawns. We have discovered that the results were variable and more complicated than we assumed in the initiation of the project. Results were to a large extent related to the morphotypic differentiation process in males. The results and the biological explanations are documented in the enclosed published article (Sagi and Ra'anan, 1988.; see Annex 1).

1.2 Establishment of an in vitro bioassay for androgenic hormones.

In this part of the study we organ-cultured in vitro the testes of Machrobrachium rosenbergii males evaluating its spermatogenic activity via the use of [³H]-thymidine as a tracer. We adjusted culture conditions (including: media, temperature, osmolarity, agitation culture dishes and sterile dissection techniques) to M. rosenbergii testes culture. Once again, we discovered that testicular activity may vary in different male morphotypes. A wide biological study was needed in order to select the optimal tissue donor for such a bioassay. The results of this search and the description of the organ culture are documented in the enclosed article (Sagi et al., 1988). These

(see Annex 2)

results were instrumental in further studying the effects of the androgenic gland peptides and ecdyson (see below) with respect to stimulation of male reproduction.

1.3 In vivo role of the androgenic gland with respect to sex characters and sex determination.

The role of the androgenic gland in vivo was elaborated by the use of androgenic gland ablation techniques (Nagamine et al., 1980). It was found that androgenic gland ablation effects may differ with accordance to temporal factors. The most important findings of this part of the study were two new functions for this gland i.e. (a) the androgenic gland plays an important regulatory role in the male morphotypic differentiation process and (b) androgenic gland factor(s) have a significant effect on the growth rate. (Sagi et. al, in press ; Annex 3).

1.4 Organ culture of testes and responsiveness to hormones

We used the organ culture system established for M. rosenbergii testes as a reproducible bioassay for the responsiveness of the tissue to androgenic hormones and molting hormones. The stimulatory effects of 20-hydroxyecdysone ^(the predominant ecdysteroid in crustaceans) on the testis are summarized in an article (enclosed) which we recently submitted for publication (Sagi et al., submitted; Annex 4) and

which stresses the nature of the cooperation between the US and Israeli laboratories. The effects of androgenic gland extracts which are being studied currently are described below (see 2.3).

2. Unpublished Results

2.1 Histological study of the androgenic gland

Androgenic glands (AG) were removed from the various male morphotypes of the freshwater prawn, M. rosenbergii. The AG were fixed in 10% formalin and stained with hematoxylin and eosin. The morphotypes ranged from small males (SM) to blue claw (BC) males. At the light microscope level, it was observed that in addition to the pyramidal clump of AG cells located at the distal portion of the vas deferens, there were significant strands of cells that extended proximally up the sperm duct for almost a centimeter.

The gland is composed of numerous thin cords of cells which are composed of ovoid cells with relatively large nuclei.

A striking difference is observable in the morphology of the AG cells between the different male morphotypes. In small males, the AG cells are spherical and densely packed (Fig. 1). There is no evidence of intercellular spaces. In weak orange claw (WOC) males, intercellular spaces appear and the cells are less densely packed (Fig. 2). This trend continues in transforming orange claw (TOC) males (Fig. 3) and is most pronounced in BC males (Fig. 4). In these latter stages, there is apparent degeneration of the gland. This may be indicative of a diminished secretion of androgenic hormone (AH).

2.2 Electron microscopy of the androgenic gland from prawn morphotypes

Initial attempts at fixing tissues in Israel and sending them to the laboratory in the USA were unsuccessful, due to the long time period between dissection and embedding. An improved protocol for the preparation of prawn tissues for electron microscopy was utilized. This was necessitated by the need to work with freshly dissected tissue from healthy animals. The tissues are first rinsed in 0.1 M phosphate buffer (pH 7.4). They are then fixed for 2 h (4% glutaraldehyde, 1% tannic acid in 0.1 M phosphate buffer, pH 7.4). The tissues are rinsed 2 times for 10 min each time in 0.1 M phosphate buffer and postfixed for 45 min in 0.5% osmium tetroxide in 0.2 M phosphate buffer. They are rinsed 3 times for 10 min each time in 0.2 M phosphate buffer. The tissues are dehydrated in an acetone series (30%, 50%, 75%, 95%, 100%, 100%, 100%, 15 min each). They are then placed into a 1:1 solution of acetone:Spurrs embedding medium overnight. The tissues are embedded in 100% Spurrs in capsules at 70°C for 24 h. Thick sections are made to determine the orientation of the tissue prior to thin sectioning by the ultramicrotome.

A series of glands were dissected and fixed. Different sections of the AG revealed an appearance that is consistent with protein secreting cells (Fig. 5). There is extensive rough ER associated with Golgi. In BC males, numerous vesicles are present. It is not clear if these are secretory vesicles or vacuoles indicative of an apparently degenerating AG of BC males (Fig. 6). Other than the degree of intercellular spacing, no

other differences were apparent between various male morphotypes.

2.3 Biochemical study of the androgenic gland

In addition to the AG, the sinus gland (SG) was obtained from the different morphotypes. The SG is a reservoir of neurosecretory endocrine material that controls molt and reproduction in crustaceans. It was hypothesized that the SG may contain material that controls the appearance of the morphotypes. The SG and AG peptide and protein hormones were examined using high-performance liquid chromatography (HPLC).

The glands were dissected from live animals and extracted in hot (80°C) HCl (0.1 N) for 5 min. The extracts were cooled, neutralized with sodium acetate, and injected directly into the HPLC. The column (4.6 x 250 mm) was packed with Vydac TP C₁₈ and eluted with a linear gradient of 0-80% solvent B (methanol) over 2 h. Solvent A was 0.1% trifluoroacetic acid in water. Flow rate was 1.0 ml/min. Detection was by absorbance at 280 nm and by post-column fluorescence (post-column solvent A was 0.1 M borate, pH 9.8; post-column solvent B was 0.02% fluorescamine in acetone). All solvents were purged with helium. This system permits the detection of as little as 5 ng of peptide.

Specific AG peptides were observed following HPLC that were not present in the surrounding sperm duct. However, the concentrations of these AG-specific peptides do not appear to differ significantly between the various male morphotypes. In addition, the neuropeptides in the SG extracts do not appear to differ significantly. Comprehensive analyses of both AG and SG

peptides were prevented due to the lack of sufficient biological material. Only freshly dissected material provided glands that were usable for HPLC analysis.

Lyophilized AG from BC males were homogenized and chromatographed on a Superose 6 (Pharmacia) FPLC (Fast protein liquid chromatography) column in the US laboratory and then shipped to Israel. Elution was with sodium phosphate (0.1 M, pH 7.2, flow rate of 7 ml/h). Fractions were collected and assayed in Israel using in vitro incorporation of radiolabeled thymidine and amino acids into cultured prawn testes. The in vitro bioassay technique was developed us in the course of the present project and included testis organ culture (as in Sagi et al., 1988; Annex 2).

and our recent study on effects of molting hormones (20-hydroxyecdysone; Sagi et al., submitted; both studies enclosed).
(Annex 4)
The separation of AG showed several peaks. We combined most of the material of interest to us into 6 fractions: I, II, III, IV, V and VI. The results (Table 1) are expressed in percent
(page 13)
increase in incorporation of radioactive material in the organ exposed to a fraction, as compared to a control (unexposed) organ from the same prawn.

This preliminary data indicated that a high molecular weight fraction of the AG extract may stimulate incorporation of both radiolabels into acid-precipitable macromolecules. Further experimentation is necessary to verify this observation.

2.4 In vitro bioassay of farnesyl acetone

Crude farnesyl acetone was obtained and purified by HPLC (60% methanol, 1.0 ml/min, C₁₈ radial compression column).

We bioassayed this putative decapod androgenic hormone (Tekitek et al., 1976) in lobsters (Homarus americanus) because of the availability of sibling juvenile animals that have been raised under controlled conditions in our culture facility at Bodega Bay. The partially purified farnesyl acetone was injected into juvenile (4th stage) lobsters. At this age the lobsters have not yet displayed any sexual dimorphism. Injections were repeated 3 times per molt cycle for 3 cycles. During the experiment, the animals were monitored daily for the appearance of male secondary sex characters and for alterations in the length of the molt cycle. Control animals were sham injected. At the end of the experiment, there were no significant differences in the ratio of the appearance of males to females in the experimental group compared to the controls. Thus it appears that the putative androgenic hormone farnesyl acetone (a lipid) is unable to induce male secondary sex characters in the lobster. Similar injection attempts together with the administration of farnesyl acetone and testosterone via food into prawns were conducted in Israel. The results were the same as found in lobster. We believe that these results are relevant to our prawn study due to the broad species specificity of a number of crustacean hormones (e.g. the molting hormone, methyl farnesoate, pigmentation-regulating hormones). These negative results have given us further impetus to continue our search for the peptide androgenic hormone. These observations are consistent with the latest reports of the isolation of a peptide androgenic hormone from isopods (Hasegawa et al., 1987).

2.5 Cell culture of crustacean testes and responsiveness to the molting hormone

We have established long term primary cell cultures from the testes of both lobsters and freshwater crayfish. These cells are viable for up to one year in a modified Medium 199 supplemented with 10% fetal bovine serum (see Table 2).

The procedure for establishing the cell cultures follows. Healthy-appearing (no lesions or lethargy), intermolt animals were surface sterilized with 20% Wescodyne for 10 min or until the animals were no longer active. Animals were rinsed with 70% ethanol and air dried in a laminar flow hood for 5 min. Aseptic carapace removal was accomplished by splitting the dorsal seam towards the rostrum and pulling each half of the carapace free of the thorax. Testes were carefully removed with thrice-flamed serrated forceps, avoiding contact with the gut and midgut gland and other sources of contamination. Testes were placed in a drop of medium (see Table 2) ^(page 14) in sterile petri dishes and minced 10-20 times with curved iris scissors or until tissue fragments were small cubes ($0.1 - 1.0 \text{ mm}^3$); excessive mincing of the tissue resulted in poor viability. To dissociate the tissue, fragments were incubated with 200 U/ml type II collagenase (Worthington) for 2-3 days at 20°C or until the testes formed smears across the bottom of the flask. The dispersed cells were pelleted twice at $50-100 \times g$ for 2 min and washed each time with fresh medium.

Penicillin (10^4 U/ml) and streptomycin (100 ug/ml) (Sigma) were added during the first month as prophylactics. Cell attachment was aided by returning the dispersed cells to the flask in which the collagenase treatment took place after the

flask was rinsed twice with media. Replicate patches of cells of equal density were achieved in the same flask by placing 50 ul of cells and medium in discrete spots in the flask. The cells adhered to the plastic in 3-4 days, at which time 2 ml of medium were added to a 25 ml flask.

This technique also worked well with plastic cover slips (12 mm diameter; Miles) placed inside the tissue flask. Cultures were fed weekly by replacing half of the spent media with fresh media.

The spermatogonial and mesodermal cells attached to the substrate within 3 days. Spermatogonial cells persisted in culture for up to one year and undergo occasional mitoses. Crayfish testicular cells persist longer than lobster testicular cells under our culture conditions. The spermatogonial cells increased in size approximately 10 fold after 2 months in culture. Mesodermal cell explants divided slowly for the first 2 weeks and then slowly degenerated over the next 6 weeks. Degeneration began with vacuole formation around the nucleus.

Interestingly, these long-term primary cultured cells are responsive to the arthropod molting hormone 20-hydroxyecdysone (20-HE). Exposure of one-month old lobster primary cell cultures to $10^{-7}M$ 20-HE caused mesodermal cells to die and spermatogonia to proliferate within one week (Figs. 7A, 7B). The hormone was withdrawn after one week and the spermatogonia continue to proliferate for 10 days (Fig. 7C).

Crayfish spermatocytes and mesodermal cells displayed minimal degeneration after one month in culture (Figs. 8A, 8C).

Exposure of these cells to 10^{-8} M 20-HE resulted in increased mesodermal cell degeneration (Figs. 8B, 8D).

These observations are consistent with those from the Israeli group using M. rosenbergii. They observed that incorporation of radiolabeled thymidine (indicative of DNA synthesis and spermatogenesis) was highest at the premolt stages of the molt cycle. This is also the time when the circulating level of 20-HE is the highest (20-HE induces the premolt condition).

Our long-term cell culture system is therefore an ideal in vitro system to examine the relationship between the molting hormone and spermatogenesis. This model system will be useful for the future examination of the endocrine regulation of spermatogenesis in the testes of the different prawn morphotypes. These data suggest a close interrelationship between molting, spermatogenesis and androgenic gland expression in the male.

By utilizing some of the animal resources at our Bodega Bay laboratory, we have been able to extend the scope of our experiments related to the elucidation of the male-determining factors in prawns. Although conducted in lobsters, the repeated demonstration of the applicability of results obtained in one crustacean system to another make our data directly relevant to the prawn project.

2.6 Demonstration of binding proteins for methyl farnesoate, a putative reproductive hormone

In insects, close relatives of crustaceans, a terpenoid hormone, juvenile hormone (JH), regulates larval development and

adult reproduction. Very recently, a JH-like molecule, methyl farnesoate (MF), has been isolated in a number of different crustaceans (Laufer et al., 1987). Data has been published that implicate a reproductive role for this molecule (Borst et al., 1987).

Due to our lack of living prawns, we used the marine shrimp Sicyonia ingentis as a model species for these studies. In addition, we utilized a novel photoaffinity analog for MF called farnesyl diazoketone (FDK). FDK was also radiolabeled. Following incubation of tissue extracts to FDK, exposure to UV light results in the formation of covalent bonds between any binding proteins and the radiolabeled FDK. These tissue extracts are then separated with denaturing polycarylamide gel electrophoresis. The gels are then exposed to X-ray film. In order to demonstrate specific binding, parallel incubations are conducted in the presence of excess unlabeled MF.

We have observed a specific binding protein in the hemolymph of the shrimp and several other crustaceans. This protein has an approximate molecular weight of about 40,000. Interestingly, this binding protein is most prevalent in early juvenile stages and in reproductive females. This protein may serve to transport MF and protect it from degradation. The differential synthesis of the MF binding protein may be an important regulatory molecule for crustacean development and reproduction. The relationship of MF and AH remains to be elucidated. It is of interest, however, that there is close structural similarity between MF and farnesyl acetone, which has been demonstrated to be biologically active in cultured testes.

Table 1. Percent increase in incorporation of radioactive material into exposed and unexposed testes of M. rosenbergii

Fraction No.	I	II	III	IV	V	VI
<u>[3 H]-thymidine</u>						
n	5	6	6	5	5	5
% increase $\pm$ SEM	+49.26 $\pm$ 54.42	+1.38 $\pm$ 12.13	+1.45 $\pm$ 25.3	-8.6 $\pm$ 17.2	+77.34 $\pm$ 31.2	+114.1 $\pm$ 30.8
<u>C-L Amino Acid</u>						
n	5	6	6	5	5	5
% increase	+39.7 $\pm$ 55.7	+10.8 $\pm$ 28.5	-5.4 $\pm$ 10.3	+52.9 $\pm$ 23.5	+52.9 $\pm$ 23.5	+61.4 $\pm$ 21.5

Table 2. Culture conditions and additions to Medium 199 buffered to atmospheric CO₂ concentrations for crayfish and lobster primary cell cultures. All cultures had 400 mg/l of NaHCO₃ and 10% fetal bovine serum added. They were maintained at 20°C at pH 7.5 (Brody and Chang, in press).

Species	Osmolarity (mosmoles)	NaCl (g/l)	Proline (mg/l)	MgCl ₂ (mg/l)
crayfish	400 - 450	2.8	40	190
lobster	950 - 1000	22.0	60	200

Figure Captions

Fig. 1. Histological section of the androgenic gland from a small male Macrobrachium rosenbergii. Note the dense packing of the cells (x20).

Fig. 2. Histological section of the androgenic gland from a weak orange claw male Macrobrachium rosenbergii. Note the limited appearance of space between the cells (x20).

Fig. 3. Histological section of the androgenic gland from a transforming orange claw male Macrobrachium rosenbergii. Note the extensive spaces between the cells and the apparent degeneration of the gland (x20).

Fig. 4. Histological section of the androgenic gland from a blue claw male Macrobrachium rosenbergii. Note the continued apparent degeneration of the gland (x20).

Fig. 5. Electron micrograph of the androgenic gland from a blue claw male. Note the extensive rough endoplasmic reticulum (x 9000).

Fig. 6. Electron micrograph of the androgenic gland from a blue claw male. Note the large amount of vesicles or vacuoles (x 7000).

Fig. 7. One-month old lobster testes primary cell culture exposed to 10^{-7} M 20-hydroxyecdysone. A. Day 0, the lower left cell group consists of spermatogonia and the upper right cell group contains mesodermal cells. B. Day 7, spermatogonia

proliferate while mesodermal cells degenerate. Hormone was then withdrawn. C. Day 14, spermatogonia continue to proliferate. Scale equals 100 um.

Fig. 8. One-month old crayfish testes primary cell culture exposed to 10^{-8} M 20-hydroxyecdysone. A. Control, day 0, mesodermal cells are to the right and spermatocytes to the left. B. Treatment, day 0, slight vacuole formation around mesodermal cell nucleus. C. Control, day 5, slight vacuole formation around mesodermal cell nucleus. D. Treatment, day 5, large vacuole formation around mesodermal nucleus. Scale equals 100 um.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

3. Summary and Conclusions

This study has made a significant contribution to the research of crustacean reproduction. We have established and utilized both in vitro and in vivo bioassays that will be instrumental in future study of shrimp and prawn reproduction.

A better understanding of the physiology of reproduction and growth of the freshwater prawn was provided by our research. The new information concerns both the establishment of monosex populations and effects of several hormones.

The two main lines of future research which seem to us most promising at this point are: (a) further purification of androgenic gland hormones and the investigation of their effect(s) with respect to sex determination and somatic growth rate. This kind of a study could use the already established bioassay and techniques and could result in increasing yields in freshwater prawn production as well as other crustaceans. (b) further steps towards establishing monosex populations on a commercial scale. The most exciting and promising results of this study, with respect to the feasibility of monosex populations of the freshwater prawn, call for further R&D steps towards commercial production of monosex populations making freshwater production profitable in temperate zones both in Israel and the USA (Sagi et al., 1986; Malecha, 1986; Cohen et al., 1988, Hulata et al., 1988).

Since BARD will not accept aquaculture proposals next year, we will be presenting our renewal proposal in 1991, based on achievements of the present study and further research which will have been conducted by then.

4. Publications Resulting from Research Project

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

Sagi, A., Milner, Y. and Cohen, D., 1988. Spermatogenesis and sperm storage in the testes of behaviorally distinctive male morphotypes of Macrobrachium rosenbergii (Decapoda, Palaemonidae). Biol. Bull., 174:330-336.

Sagi, A. and Z. Ra'anan, 1988. Morphotypic differentiation of males of the fresh-water prawn Macrobrachium rosenbergii: changes in the midgut glands and the reproductive system. J. Crust. Biol., 8:43-47.

Sagi, A., Cohen, D. and Y. Milner, 1989. Effect of androgenic gland ablation on morphotypic differentiation and sexual characteristics of male freshwater prawns Macrobrachium rosenbergii. Gen. Comp. Endocrinol., in press.

Sagi, A., Karp, L., Milner, Y., Cohen, D., Kuris, A.M. and E. Chang. Variation in spermatogenesis during the molt cycle of Macrobrachium rosenbergii (Decapoda, Palaemonidae) - direct effect of 20-Hydroxyecdysone in vitro., submitted for publ.

5. Other Related Literature

Borst, D.W., Laufer H., Landau M., Chang E., Hertz W.A., Baker F.C. and D.A. Schooley, 1987. Methyl farnesoate (MF) and its role in crustacean reproduction and development. Insect Biochem., 17:1123-1127.

Brody, M.D. and E.S. Chang, 1989. Development and utilization of crustacean long-term primary cell cultures: ecdysteroid effects in vitro. Invert. Repr. and Develop., in press.

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Sagi, A., Ra'anan, Z., Cohen, D. and Y. Wax, 1986. Production of Macrobrachium rosenbergii in monosex populations: yield characteristics under intensive monoculture conditions in cages. Aquaculture, 51: 265-275.

Tekitek, A., Berreur-Bonnenfant, J., Ferezou, J.P., Meusy, J.J., Barbier, M. and E. Lederer, 1976. Methylations biologiques chez le crabe Carcinus maenas; inhibition in vitro par une fraction purifiée extraite des glands androgènes. Biochimie 58:1355-1358.