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DEVELOPMENT OF A PROTEIN-FREE CHEMICALLY DEFINED CULTURE MEDIUM FOR THE PROPAGATION OF THE OYSTER PATHOGEN PERKINSUS MARINUS

LA PEYRE J.F.* & FAISAL M.*

Summary:
In the present study we describe a protein-free, chemically defined culture medium, designated JL-ODRP-3, which supports the propagation of Perkinsus marinus, a parasite of the eastern oyster, Crassostrea virginica. P. marinus adapted rapidly to the defined medium and the growth rate of the protozoan increased significantly following a few subcultures. Two isolates of P. marinus, one from the Chesapeake Bay (Virginia) and the other from the Gulf of Mexico (Texas) were cultured for at least ten passes. The doubling times for the isolates from Virginia and Texas, in log phase, were 18 ± 1.2 and 28.6 ± 3.2 hours respectively, after ten passes in JL-ODRP-3. Moreover, P. marinus cells cultured in the defined medium were infective to eastern oysters. Finally, the defined medium was used successfully to initiate continuous cultures of P. marinus from heart fragments of oysters. The absence of proteins and peptides from this chemically defined medium demarcates JL-ODRP-3 as the most suitable medium to study P. marinus proteins, to produce antigens for antibody production, and to screen chemotherapeutic agents.

KEY WORDS: Perkinsus marinus, protozoa, in vitro, protein-free chemically defined culture medium, oyster parasite, Crassostrea virginica.

INTRODUCTION
The protozoan Perkinsus marinus causes heavy losses in oyster populations of the Atlantic and Gulf coasts of the United States (reviewed in Burreson and Calvo, 1996; Ford, 1996; Soniat, 1996). This oyster pathogen was discovered in the Gulf of Mexico in 1948 (Mackin et al., 1950) and named Dermocystidium marinus as it was thought to be a fungus. Since Perkins (1976) showed that the motile stage of P. marinus, the zoospore, contained an apical complex, the parasite was renamed and included in the phylum Apicomplexa (Levine, 1978).

Following four decades of trials to culture P. marinus, it was recently demonstrated that P. marinus could be propagated in vitro (reviewed in La Peyre, 1996). The culture medium used for the first isolation of P. marinus, JL-ODRP-1, consisted of more than 60 ingredients combined to resemble the known composition, osmolality, and pH of plasma of bivalve mollusks (La Peyre et al., 1993). This medium was supplemented with purified fraction V of bovine serum albumin (BSA), cod liver oil, and yeastolate ultrafiltrate (10 kDa). The use of animal serum was avoided to eliminate the effects of undefined complex mixtures on P. marinus (Barnes and Sato, 1980; Maurer, 1992). In order to identify and purify proteins of P. marinus, BSA was eliminated from JL-ODRP-1 and culture conditions were optimized (La Peyre and Faisal, 1996). Using this protein deficient medium, multiple parasite extracellular proteins including serine proteases were identified (La Peyre et al., 1995).

The BSA-free JL-ODRP-1 medium, however, contains the undefined ingredients yeastolate and cod liver oil,
both of which could interfere with a variety of experiments. For example, peptides in the yeastolate ultrafiltrate can potentially inhibit the uptake of certain chemotherapeutic agents (Kerridge and Vanden Bossche, 1990). The presence of yeastolate ultrafiltrate in the culture medium has also induced polyclonal mitogenesis in mouse splenocytes, thus interfering with the development of monoclonal antibodies against *P. marinus* extracellular proteins (Faisal et al., 1996). In this study we report the composition of the protein-free, chemically defined culture medium, JL-ORDP-3. We also provide evidence that JL-ORDP-3 supports the propagation of established *P. marinus* cell lines, is suitable for the initiation of primary cultures of *P. marinus* from naturally infected oysters, and that following many subcultures in this medium, *P. marinus* remains infective to eastern oysters.

**MATERIALS AND METHODS**

**MODIFICATIONS OF THE CULTURE MEDIUM JL-ORDP-1**

The protein-free chemically defined medium was modified from JL-ORDP-1 medium (La Peyre et al., 1993) by elimination of BSA, yeastolate ultrafiltrate and cod liver oil, by increasing the amino acid and vitamin concentrations and by adding a chemically defined lipid solution and vitamin B12. A detailed procedure to prepare this chemically defined medium, designated JL-ORDP-3, is provided below and its final composition is given in Table I. The pH of the chemically defined medium was 7.4 and osmolality was 754 (± 4) mOsm/kg as measured with a 5,500 vapor pressure osmometer (Wescor Inc., Logan, UT). No adjustment in the pH or osmolality of JL-ORDP-3 was necessary.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>mg/L</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major inorganic salts and buffers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium Chloride Anhydrous</td>
<td>199.8</td>
<td>S</td>
</tr>
<tr>
<td>Magnesium sulfate Anhydrous</td>
<td>3,371.3</td>
<td>S</td>
</tr>
<tr>
<td>Magnesium Chloride Anhydrous</td>
<td>2,031.3</td>
<td>S</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>574.6</td>
<td>S</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>15,973.3</td>
<td>S</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>2,000.0</td>
<td>S</td>
</tr>
<tr>
<td>HEPES</td>
<td>5,957.5</td>
<td>S</td>
</tr>
<tr>
<td><strong>Trace elements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.4</td>
<td>S</td>
</tr>
<tr>
<td>Sodium Bromide</td>
<td>0.686</td>
<td>S</td>
</tr>
<tr>
<td>Sodium Fluoride</td>
<td>0.2799</td>
<td>S</td>
</tr>
<tr>
<td>Strontium Chloride Hexahydra</td>
<td>17.77</td>
<td>S</td>
</tr>
<tr>
<td>Cupric Sulfate 7H₂O</td>
<td>0.00249</td>
<td>S</td>
</tr>
<tr>
<td>Ferrous Sulfate 5H₂O</td>
<td>0.854</td>
<td>S</td>
</tr>
<tr>
<td>Zinc Sulfate 7H₂O</td>
<td>0.1488</td>
<td>S</td>
</tr>
<tr>
<td>Manganese Sulfate H₂O</td>
<td>0.0000338</td>
<td>G</td>
</tr>
</tbody>
</table>

**Amino Acids**

- L-Arginine .HCl 63.2 G
- L-Cystine 12.0 G
- L-Glutamine 50.0 G
- L-Histidine HCl H₂O 21.0 G
- L-Leucine 26.2 G
- L-Isoleucine 26.26 G
- L-Lysine HCl 36.26 G
- L-Methionine 7.56 G
- L-Phenylalanine 16.5 G
- L-Threonine 23.8 G
- L-Tryptophan 3.1 G
- L-Tyrosine 18.0 G
- L-Valine 23.4 G
- L-Alanine 108.9 G
- L-Asparagine H₂O 15.0 G
- L-Aspartic Acid 13.3 G
- L-Glutamic Acid 14.7 G
- L-Glycine 57.5 G
- L-Proline 11.5 G
- L-Serine 60.5 G
- Taurine 150.0 G

**Carbohydrates**

- Glucose 500.0 S
- Galactose 100.0 S
- Trehalose 100.0 S

**Nucleic Acid Precursors**

- Adenosine 5'-Monophosphate 1.0 S
- Cytidine 5'-Monophosphate 1.0 S
- Uridine 5'-Triphosphate 1.0 S

**Vitamins**

- D-Ca Pantothenate 0.4 S
- Choline Chloride 3.0 S
- Folic Acid 2.0 S
- L-Inositol 35.0 S
- Nicinamide 2.0 S
- P-AminoBenzoic Acid 2.0 S
- D-Pantothenic Acid 0.5 S
- Pyridoxin HCl 2.0 S
- Riboflavin 0.4 S
- Thiamine HCl 2.0 S
- Vitamin B-12 0.01 S

**Lipids**

- Arachidonic Acid 0.07 G
- Cholesterol 2.20 G
- DL-a-Tocopherol-Acetate 0.7 G
- Linoleic Acid 0.1 G
- Linolenic Acid 0.1 G
- Myristic Acid 0.1 G
- Oleic Acid 0.1 G
- Palmitic Acid 0.1 G
- Palmitic Acid 0.1 G
- Stearic Acid 0.1 G

**Miscellaneous**

- Coenzyme A 1.0 S
- Phenol Red 1.0 S
- Chloramphenicol 5.0 S
- Ethyl alcohol 1,000.0 G
- Pluronic® F-68 1,000.0 G
- Tween 80 2,000.0 G

*Source of ingredients: G = Gibco laboratories, Grand Island, NY; S = Sigma Chemical Co., St Louis, MO.

Table I. — Composition of the medium JL-ORDP-3. Osmolality 756 mOsm/Kg, pH 7.4.
**PERKINSUS MARINUS IN A PROTEIN-FREE DEFINED MEDIUM**

All chemicals used were tissue culture grade reagents purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. A basal salt solution (BSS) was first prepared by dissolving 22 g of Seawater Synthetic Basal Salt Mixture and 2 g sodium bicarbonate in a final volume of 912 ml of culture grade water (Milli-Q UF water purification system, Millipore Co. Bedford, MA). Potassium chloride (0.1772 g) was added to maintain a Na⁺/K⁺ ratio of 23. The BSS solution was then filtered (0.22 μm) sterilized. HEPES (25 ml of a 1 M solution) was added for buffering at a 5% CO₂ atmosphere. The following solutions were then added to BSS under sterile conditions:

1) 2 ml of Trace Element Mix (Gibco BRL, Grand Island, NY),
2) 1 ml of a solution containing 0.834 mg/ml of ferrous sulfate and 0.143 mg/ml of zinc sulfate,
3) 1 ml of a solution containing 0.249 mg/ml of cupric sulfate,
4) 10 ml of MEM Amino Acids Solution without glutamine (Gibco BRL),
5) 10 ml of MEM Non-Essential Amino Acids Solution (Gibco BRL),
6) 10 ml of a solution containing 10 mg/ml of alanine, 5 mg/ml of glycine, 5 mg/ml serine, 15 mg/ml taurine and 5 mg/L glutamine,
7) 6 ml of RPMI 1640 Vitamin Solution,
8) 10 ml of a carbohydrate solution containing 50 mg/ml of glucose, 10 mg/ml of trehalose, and 10 mg/ml of galactose,
9) 10 ml of Chemically Defined Lipid Concentrate (Gibco BRL),
10) 2 ml of a solution containing 0.5 mg/ml each of adenosine 5'-monophosphate, cytidine 5'-monophosphate, uridine 5'-triphosphate and coenzyme A,
11) 10 ml of a solution containing 0.5 mg/ml of chloramphenicol,
12) 1 ml of Phenol red solution (Gibco BRL).

**DETERMINATION OF THE CAPABILITY OF JL-ORDP-3 TO SUPPORT THE PROPAGATION OF ESTABLISHED P. MARINUS CELL LINES**

Two isolates of *Perkinsus marinus*, *Perkinsus*-1 and LMTX-1, were used to demonstrate the capability of JL-ORDP-3 to support their propagation in vitro. *Perkinsus*-1 is the first isolate of *P. marinus* to be successfully propagated in vitro. This cloned and fully characterized isolate of *P. marinus* was obtained from the heart of an infected oyster from the lower Chesapeake Bay, in February of 1992, and has been subcultured every two to four weeks in JL-ORDP-1 medium (La Peyre et al., 1993). The medium JL-ORDP-1 was slightly modified and contained 4 mg/ml instead of 12 mg/ml of BSA after June 1993. The isolate LMTX-1 was kindly provided by Dr. Dave Bushek (Belle W. Baruch Institute of Marine Biology and Coastal Research, University of South Carolina) in June 1994. Culture of LMTX-1 was initiated in July 1993 from hypnosporous of an infected oyster collected in Laguna Madre, Texas using a technique described by La Peyre and Faisal (1995), Bushek (1994). This isolate has also been propagated in JL-ORDP-1. Division of both isolates propagated in JL-ORDP-1 medium is by schizogony.

Stock cultures of *Perkinsus*-1 and LMTX-1 were propagated in 75 cm² flasks (Corning Glassworks, Corning, NY) using the JL-ORDP-1 medium (50 ml/flask) at a seeding density of 10⁶/ml, and subcultured every four weeks. All culture flasks were maintained at 28 °C in a humidified chamber in the presence of 5% CO₂ tension. *Perkinsus marinus* cells were harvested, resuspended in 50 ml of either medium and triplicate 75 cm² culture flasks at a seeding density of 2 x 10⁷ cells/ml. Samples (1.5 ml/flask) of cultured media were collected on days one, three, five, seven, nine, twelve and fifteen for cell counts with a Bright-line hemacytometer (Reichert, Buffalo, NY). Cell clumps arising from division of the schizonts were disaggregated by three passages through a 25-gauge needle prior to counting. The viability of the cells was determined by staining with 0.005% of neutral red.

The growth of *P. marinus* cells in JL-ORDP-3 medium during the first subculture was compared to growth in JL-ORDP-1. *Perkinsus marinus* cells in each medium were then subcultured every three weeks and their growth was followed for the first three consecutive subcultures (i.e., 2nd to 4th subculture) and at the eleventh subculture as previously described.

**INFECTION OF OYSTERS WITH P. MARINUS CELLS PROPAGATED IN JL-ORDP-3**

To determine whether *P. marinus* would retain its infectivity to eastern oysters following several subcultures in the chemically defined medium, *P. marinus*-free oysters (Pemaquid Oyster Co., Waldoboro, Maine) were experimentally infected with cultured cells. *Perkinsus*-1 cells propagated in JL-ORDP-3 (18th subculture) were harvested, resuspended at a density of 10⁷ cells/ml of an iso-osmotic salt solution (27 ppt, ~750 mOsm, Forty Fathoms marine mix, Marine Enterprises Inc., Baltimore, MD) and 100 μl of this suspension was injected into the mantle cavity of 25 notched oysters. After 14 days, oysters received a second dose of *Perkinsus*-1 (10⁶ cells/oyster). Control oysters (25) received the same regimen of a salt solution. Each oyster group was maintained in an aerated 80 liter tank.
at 28 °C. The water was changed weekly with 1 μm filtered estuarine water (York River, Gloucester point, VA) adjusted to 27 ppt with Forty Fathoms marine mix salts. The oysters were not fed to avoid the introduction of extraneous P. marinus cells via algae. Forty-two days post-infection, the presence of P. marinus cells in both oyster groups was determined in the rectal, gill and mantle tissue of each individual oyster using the Ray’s Test (Ray, 1952). The intensity of infection in each oyster was rated according to the categories of Ray (1954) by estimating the percentage of tissue occupied by the parasite.

**Suitability of JL-ORDP-3 to support primary cultures of P. marinus from naturally infected oysters**

*Perkinsus marinus*-infected eastern oysters were collected from Wreck Shoal, James River (VA). Cultures of *P. marinus* were initiated from the heart fragments of ten infected oysters according to the procedure described in La Peyre et al. (1993). Briefly, the heart from each oyster was removed aseptically, rinsed in a concentrated antibiotic solution and finely minced with a razor blade. The heart fragments were then washed five times in a sterile filtered (0.2 μm) salt solution, resuspended in JL-ORDP-3 medium, and placed in 25 cm² culture flasks. The cultures were incubated at 28 °C in the presence of 5 % CO₂ atmosphere. Propagation of *P. marinus* was monitored using an Olympus (CK-2) inverted light microscope with phase contrast optics at a magnification of 200 ×. After three weeks of incubation, subculturing was attempted.

**Statistical analysis**

Growth rate was expressed as doubling time (i.e., the time for a population to double in number) during log phase. In this study, log phase was defined as the period of maximum increase in cell number between two sampling time. Doubling time data was log₁₀ transformed and compared by one factor analysis of variance followed by SNK’s multiple comparisons of means when significant differences (p < 0.05) were found.

**Results**

**Propagation of P. marinus established cell lines in JL-ORDP-3**

*Perkinsus*-1 cells proliferated in JL-ORDP-3 following their transfer from JL-ORDP-1 medium, however, the growth rate in JL-ORDP-3 was significantly lower than that of the same stock of cells grown in JL-ORDP-1 (Fig. 1). The doubling time for *Perkinsus*-1 cells in JL-ORDP-3 was about 22 ± 1.4 hours which is significantly greater than that of cells cultured in JL-ORDP-1 (i.e., 15 ± 0.8 hours, Table II). Viability of *Perkinsus*-1 cells in defined medium after the initial transfer from JL-ORDP-1 was always > 95 %. The growth rate of *Perkinsus*-1 increased between the first and second subculture and did not significantly change after 11 subcultures (Fig. 1, Table II).

The medium JL-ORDP-3 also sustained the propagation of LMTX-1 cells after a period of adaptation. Cells of LMTX-1 did not initially multiply following their transfer and their viability was reduced to 78 %. LMTX-1 required a longer time to adapt to JL-ORDP-3. The growth rate increased progressively from the second to the eleventh subculture (Fig. 2, Table II). The viability of cultured LMTX-1 cells after the 11th subculture rose to > 95 %.

**Morphology of P. marinus in JL-ORDP-3**

Microscopical examination of *Perkinsus*-1 and LMTX-1 cells cultured in JL-ORDP-3 medium revealed that their morphology was alike the reported morphology of *P. marinus* observed either in vivo or freshly isolated.

Fig. 1. — Propagation of *Perkinsus*-1 in JL-ORDP-1 (48th subculture) and in JL-ORDP-3 media (1st, 4th and 11th subcultures following transfer from JL-ORDP-1). Culture flasks (N = 3) were seeded with 2 x 10⁵ cells/ml and were incubated at 28° C under 5 % CO₂ tension.
Table II. — Doubling times of *Perkinsus*-1 and LMTX-1 cells subcultured in JL-ORDP-1 and in the protein-free defined medium JL-ORDP-3. Cells were seeded into 50 cm² flasks at a density of 2 x 10⁵ cells/ml.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Medium</th>
<th>Subculture</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Perkinsus</em>-1</td>
<td>JL-ORDP-1</td>
<td>45h</td>
<td>15.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>JL-ORDP-3</td>
<td>1st</td>
<td>22.3 ± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2nd</td>
<td>17.1 ± 2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3rd</td>
<td>16.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4th</td>
<td>16.9 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11th</td>
<td>18.0 ± 1.2</td>
</tr>
<tr>
<td>LMTX-1</td>
<td>JL-ORDP-1</td>
<td>16h</td>
<td>23.8 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>JL-ORDP-3</td>
<td>1st</td>
<td>np**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2nd</td>
<td>50.4 ± 3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3rd</td>
<td>35.3 ± 7.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4th</td>
<td>37.1 ± 4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11th</td>
<td>28.6 ± 3.2</td>
</tr>
</tbody>
</table>

* Calculated during log phase.
** np indicates no propagation.

*Perkinsus marinus* isolated from infected heart fragments propagated in the defined medium. During the first three weeks of primary culture, protozoal cells showed the typical morphology of *P. marinus*, enlarged, and divided by schizogony. Subculturing was attempted at a density of 10⁶ cells/ml and continuous growth were obtained from eight out of ten oysters. The remaining heart cells did not divide and were diluted out after a few subcultures.

**DISCUSSION**

*Perkinsus marinus* could be propagated in the protein-free culture medium JL-ORDP-3. It was important to subculture *P. marinus* cells for at least eleven passages in JL-ORDP-3 medium in order to eliminate low concentrations of chemical ingredients that may have been taken up by the cells while growing in JL-ORDP-1 and, to acclimate protozoal cells to the new medium. As our results demonstrate, JL-ORDP-3 supported the continuous growth of two *P. marinus* isolated through at least ten subcultures. The growth rates measured for both *P. marinus* isolates, *Perkinsus*-1 and LMTX-1, in the defined medium were lower than in the original medium JL-ORDP-1, however their doubling times were within the range reported for *P. marinus* grown in protein supplemented media (Gauthier and Vasta, 1995; Dungan and Hamilton, 1995). The decreased growth rates can be attributed to the absence of proteins, peptides, and possibly growth factors that may have been present in the original medium undefined components. Our previous studies have demonstrated that proteins present in JLORDP-1 medium were consumed by *P. marinus* (La Peyre and Faisal, 1995) that produces multiple serine proteases (La Peyre et al., 1995).
Fig. 3. – Light micrographs of Perkinsus-1 and LMTX-1 cells after 11th subculture in JL-ORDP-3 medium. Flasks were seeded with 2 × 10^5 cells/ml and maintained at 28°C under 5% CO₂. A) small-sized Perkinsus-1 cells: notice the refractile lipid bodies (bar = 10 µm); B) larger Perkinsus-1 cells with clear vacuole (bar = 10 µm); C) enlarged LMTX-1 cell dividing by schizogony (bar = 25 µm).

Fig. 4. – Light micrographs of Perkinsus-1 cells after 17th subculture in JL-ORDP-3 medium. Flasks were seeded with 2 × 10^5 cells/ml and maintained at 28°C in 5% CO₂ for one week prior to photographing. A) Perkinsus-1 mother cells yielding two daughter cells (bar = 25 µm); B) Perkinsus-1 mother cell dividing (bar = 10 µm); C) cell wall (arrow) of a mother cell following division by schizogony which yielded two daughter cells (bar = 10 µm).

The morphology of Perkinsus-1 and LMTX-1 cells in JL-ORDP-3 was similar to the morphology of P. marinus observed either in vivo or freshly isolated from infected oysters (Mackin et al., 1950; Perkins, 1969, 1976; La Peyre and Chu, 1994). The size of the smaller cells (i.e., 3-6 µm) was identical to the size of merozoites isolated from infected oysters by La Peyre and Chu (1994). The small cells had prominent refractile bodies, presumably lipid droplets, like those observed in isolated merozoites. Moreover, the size of the largest dividing cells never exceeded about 30 µm which is the size of P. marinus schizonts in vivo (Perkins, 1969). On the other hand, cells cultured in JL-ORDP-1 had a much larger size that sometimes exceeded 45 µm in diameter (La Peyre et al., 1993). The high protein concentration (12 mg/ml BSA) in the original medium JL-ORDP-1 may have caused greater enlargement of cultured cells. Division of P. marinus cells of both isolates in JL-ORDP-3 was by schizogony. Mother cells yielded from two to many daughter cells (presumably 4-32).

A defined medium has recently been developed by Gauthier et al. (1995). This medium consists of Dulbecco modified Eagle's medium/Ham's F12 nutrient mixture (1:1) supplemented with 1.7 mg/ml of fetuin. The major advantage of this medium is its ease of preparation since each component is commercially available. However, fetuin and fetuin breakdown products could interfere with studies on P. marinus-derived proteins and peptides. All other commercial media that have been used to culture P. marinus were supplemented with fetal bovine serum (FBS, 5-20%) alone or with oyster plasma (5-20%) (Gauthier and Vasta, 1993; Kleinshuster and Swink, 1993; Dungan and Hamilton, 1995).

Results of this study clearly demonstrated that despite the absence of proteins, JL-ORDP-3 supported the propagation of P. marinus in vitro and the initiation of primary cultures. The propagated cells retained their infectivity and were identical in their morphology to P. marinus in vivo. The absence of proteinaceous materials from this chemically defined medium demarcates JL-ORDP-3 as the most suitable medium to study P. marinus proteins, to produce antigens for antibody production, and to screen chemotherapeutic agents. Moreover this medium will greatly simplify biochemical, physiological and nutritional studies of P. marinus.
ACKNOWLEDGEMENTS

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