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APPENDICES

APPENDIX A

ORIGINAL DATA FROM COMPETITIIVE ELISA

Table 1A VTG and ZRP concentrations of Greenback Mullet (*Liza subviridis*) juvenile fish serum, which were injected by various estradiol (E2) concentration: 1-4; 0 , 2.5 , 5 and 50 mg of estradiol (E_2)/kg body weight respectively.

sample	Lt (cm)	Wt (g)	Total serum (ml)	VTG Levels (mg/ml)	ZRP Levels (mg/ml)
T1-1	20.4	85	0.75	0.052	0.115
T1-2	17	50	0.3	0.000	0.000
T1-3	20.8	95	0.25	0.017	0.080
T1-4	29	280	1.3	0.022	0.096
T1-5	29.2	265	1	0.064	0.079
T1-6	30	275	0.8	0.037	0.871
T1-7	22.4	120	0.7	0.020	0.146
T1-8	22	115	1	0.044	0.096
T1-9	24.6	150	0.7	0.017	0.204
T1-10	23.2	120	0.7	0.000	0.078
T1-11	20.6	75	0.75	0.000	0.000
T1-12	18.1	50	0.25	0.017	0.097
T1-13	16	45	0.75	0.000	0.091
T1-14	20.3	86	0.75	0.000	0.148
T1-15	22	105	0.75	0.051	0.11
T1-16	21	85	1	0.028	0.801
T1-17	11.8	15	0.4	0.000	0.000
T1-18	15.4	25	0.6	0.000	0.071
T1-19	12	15	0.5	0.000	0.098
T1-20	15.8	40	0.25	0.000	0.098
T1-21	15.9	40	0.5	0.051	0.161

sample	Lt (cm)	Wt (g)	Total serum (ml)	VTG Levels (mg/ml)	ZRP Levels (mg/ml)
T1-22	15.2	30	0.5	0.015	0.018
T1-23	19.6	80	0.5	0.000	0.098
T1-24	17.6	50	0.6	0.000	0.173
T1-25	14.6	30	0.45	0.000	0.145
T2-1	15.5	35	0.8	0.637	2.276
T2-2	18.5	50	0.5	0.598	1.398
T2-3	16.6	35	0.5	0.503	1.595
T2-4	22.5	100	0.5	0.247	1.272
T2-5	21.2	105	0.6	0.572	3.643
T2-6	16.7	45	0.5	0.275	0.313
T2-7	18.3	50	0.5	0.194	0.407
T2-8	23	100	1	0.551	1.980
T2-9	26.5	140	0.5	0.627	2.012
T2-10	27	160	0.8	0.609	3.614
T2-11	21	100	1.2	0.554	2.004
T2-12	17	45	0.6	0.010	0.661
T2-13	18	50	0.8	0.000	0.144
T2-14	22	110	0.8	0.545	1.291
T2-15	22	90	0.9	0.371	0.913
T2-16	19	70	0.4	0.000	0.130
T2-17	18	55	0.7	0.456	0.892
T2-18	17	55	1	0.564	1.148
T3-1	18	50	0.7	1.715	3.391
T3-2	20	60	0.8	1.739	4.487
T3-3	19.5	70	0.85	1.717	3.465
T3-4	19.5	60	0.5	1.860	4.211
T3-5	19	55	0.7	1.713	3.023
T3-6	20	60	0.6	1.601	2.814
T3-7	19	53	0.5	1.744	4.076

sample	Lt (cm)	Wt (g)	Total serum (ml)	VTG Levels (mg/ml)	ZRP Levels (mg/ml)
T3-9	19	65	1	1.816	3.022
T3-10	16	40	0.75	1.558	4.475
T3-11	18	45	0.7	1.659	5.261
T3-12	19	50	0.75	1.650	4.475
T3-13	21	70	0.5	1.563	4.680
T3-14	17.5	45	0.7	1.582	3.391
T3-15	15.5	30	0.75	1.588	3.475
T4-1	17	45	1	2.065	3.495
T4-3	14.8	20	0.5	2.010	2.275
T4-4	14	20	0.4	1.961	1.793
T4-5	17.7	45	0.7	2.199	2.864
T4-6	19	55	0.8	1.931	2.360
T4-8	17.5	45	0.65	2.028	2.012
T4-9	11	20	0.5	1.843	1.974
T4-10	19.3	50	0.75	2.123	2.772
T4-12	14	23	0.4	1.971	2.951
T4-13	14.5	25	0.6	2.008	2.012
T4-14	14	25	0.25	2.388	2.070
T4-15	15.5	26	0.4	1.961	1.398
T4-16	19.8	65	0.7	2.014	1.252
T4-17	18.5	50	0.5	2.011	2.153
T4-18	16.5	40	0.6	1.091	1.643
T4-19	16	30	0.4	2.009	1.275
T4-20	20	65	0.4	2.293	0.706
T4-21	16	30	0.5	2.079	1.012
T4-22	18	40	0.5	2.388	1.313
T4-23	15.5	30	0.5	2.386	1.012
T4-24	18	40	0.5	2.360	1.133
T4-25	16	30	0.6	2.068	1.398

sample	Lt (cm)	Wt (g)	Total serum (ml)	VTG Levels (mg/ml)	ZRP Levels (mg/ml)
T4-26	18	40	0.7	2.293	1. 596
T4-28	16	30	0.4	2.179	1.253
T4-29	15	20	0.5	2.018	2.643

APPENDIX B

BUFFER AND REAGENT PREPARATION

1. Phosphate buffered saline (PBS) 0.15 M pH 7.2

NaCl	8.0	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g
Or $Na_2HPO_4 . 7H_2O$	2.15	g
H_2O (distilled water) adjust volume to	1000.0	ml

2. 5% Blotto solution (Johnson et al., 1984)

Skimmed milk	5.0	g
PBS 0.15 M pH 7.2	100.0	ml
1% Merthiolate (Sigma)	1.0	ml
Triton X-100 (Sigma)	0.1	ml

3. 1% Merthiolate

Thimerosal (Sigma)	1.0	g
H_2O (distilled water) adjust volume to	100.0	ml

APPENDIX C

REAGENT PREPARATION FOR HYBRIDOMA PRODUCTION

1. RPMI medium

The medium consisted of the following ingredients:

Ingredient	Amount	Unit
RMPI 1640 (Roswell Park Memorial Institute – Gibco BRL, USA)	10.4	g
D-glucose (Sigma)	3.6	g
L-glutamine (Sigma)	0.2923	g
Sodium pyruvate ($C_3H_3O_3Na$) (Sigma)	1.1005	g
$NaHCO_3$	2.0160	g
HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma)	5.9525	g
H_2O (Meri Q water)	1000.0	ml

The solution of penicillin G, streptomycin and kanamycin were added to the final concentration of 20,000 units, 200 mg and 200 mg per liter, respectively. The medium was sterilized by millipore membrane (pore size 0.22 μm) filtration and stored at 4 °C.

2. RPMI medium with serum

The medium consisted of the following ingredients:

Ingredient	Amount	Unit
RPMI medium (1)	80.0	ml
Fetal calf serum (FCS, Starrate, Australia)	20.0	ml
Or Bovine calf serum (BCS, Starrate, Australia)		
100 x HT supplement (Gibco BRL, USA)	1.0	ml
-10 mM Sodium hypoxanthine		

-1.6 mM Thymidine

3. Hybridoma selective medium (HAT medium)

The medium consisted of the following ingredients:

RPMI medium (1)	80.0	ml
FCS	20.0	ml
HT supplement	1.0	ml
50 X Aminopterin (Sigma)	2.0	ml
1 % Mouse red blood cell		

4. Fusion solution (40% polyethylene glycol)

Polyethylene glycol (PEG)	4.0	g

The solution was prepared by dissolving 4.0 g of polyethylene glycol in 6 ml of RPMI medium (1). The solution was incubated at 37 °C in CO_2 incubator before use.

5. Freezing medium (12% DMSO)

Dimethylsulfoxide (DMSO, Sigma)	12.0	ml
RPMI medium (1)	88.0	ml

The medium was stored in cold (4 °C) before use.

APPENDIX D

BUFFER AND SOLUTION FOR SODIUM DODECYL SULFATE POLYCRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOT ANALYSIS

1. Stock solution

1.1 Monomer solution (30 %T, 2.7% C_{Bis})

Acrylamide (BIO-RAD)	58.4	g
Bis (N,N'-methylene-bis-acrylamide, BIO-RAD)	1.6	g
H_2O (distilled water) adjust volume to	200.0	ml

Stored at 4 °C in dark bottle.

1.2 4 X Running gel buffer (1.5 M tris-Cl pH 8.8)

Tris (hydroxymethyl) aminomethane (BIO-RAD)	36.3	g
H_2O (distilled water) adjust volume to	200.0	ml

Adjusted pH with HCl

1.3 4 X Stacking gel buffer (0.5 M tris-Cl pH 6.8)

Tris	3.0	g
H_2O (distilled water) adjust volume to	50.0	ml

Adjusted pH with HCl

1.4 10% SDS

SDS (sodium dodecyl sulfate, BIO-RAD)	50.0	g
H_2O (distilled water) adjust volume to	500.0	ml

1.5 10 % Ammonium persulfate (freshly prepared)

Ammonium persulfate (BIO-RAD)	0.1	g
H_2O (distilled water)	1.0	ml

1.6 Running gel overlay (0.375 M tris-Cl pH 8.8, 0.1% SDS)

1.5 M Tris (1.2)	25.0	ml
10% SDS (1.4)	1.0	ml
H_2O (distilled water) adjust to	100.0	ml

1.7 2 X Treatment buffer (0.125 M tris-Cl pH 6.8,4% SDS, 20% glycerol, 10% 2-mercaptoethanol)

0.5 M Tris (1.3)	2.5	ml
10% SDS (1.4)	4.0	ml
Glycerol	2.0	ml
2-Mercaptoethanol	1.0	ml
H_2O (distilled water)	0.5	ml

2. Preparation of separating gel and stacking gel

2.1 Separating gel for SDS-PAGE 15% gel (15 % T 2.7 % C_{BIS})

Monomer solution (1.1)	15.0	ml
1.5 M tris-Cl (1.2)	7.5	ml
10 % SDS (1.4)	0.3	ml
H_2O (distilled water)	6.75	ml
10% Ammonium persulfate (1.5)	150.0	µl
TEMED	20.0	µl

2.2 Stacking gel for SDS-PAGE 4% gel (4% T 2.7% C_{BIS})

Monomer solution (1.1)	2.66	ml
1.5 M tris-Cl pH 6.8 (1.3)	5.0	ml
10 % SDS (1.4)	0.2	ml
H_2O (distilled water)	12.2	ml

10% Ammonium persulfate (1.5)	100.0	µl
TEMED	10.0	µl

Table 1E Preparation of separating gel and stacking gel

	Separating gel	Stacking gel
	15 % T 2.7% C_{BIS} (for SDS-PAGE)	4% T 2.7 % C_{BIS}(for SDS-PAGE)
30 % T 2.7 % C_{BIS}	15.0 ml	2.66 ml
1.5 M tris-Cl pH 8.8(1.2)	7.5 ml	-
0.5 M tris-Cl 6.8 (1.3)	-	5.0 ml
10 % SDS	0.3 ml	0.2 ml
H_2O	6.75 ml	12.2 ml
Mixed and deaerated using vacuum pump		
10 % Ammonium persulfate (1.5)	150 µl	100 µl
TEMED	20 µl	10 µl
Mixed and rapidly poured between the glass plate		

3. Running buffer

3.1 SDS-PAGE Tank buffer (0.025 M tris pH 8.3, 0.192 M glycine, 0.1% SDS)

Tris	12.0	g
Glycine	57.6	g

10% SDS (1.4)	40.0	ml
H_2O (distilled water)	4000.0	ml

4. Staining and destaining solution

4.1 Staining solution for protein (Coomassie blue)

4.1.1 Stain stock (1% Coomassie blue R-250)

1% Coomassie blue R-250	1.0	g
H_2O (distilled water)	100.0	ml

4.1.2 Stain (0.1% Coomassie blue R-250, 50% methanol, 10% acetic acid)

Stain stock (4.1.1)	50.0	ml
Methanol	250.0	ml
Acetic acid	50.0	ml
H_2O (distilled water) adjust to	500.0	ml

4.2 Destaining solution for Coomassie blue

4.2.1 Destain I (50% methanol, 10% acetic acid)

Methanol	500.0	ml
Acetic acid	100.0	ml
H_2O (distilled water) adjust to	1000.0	ml

4.2.2 Destain II (5% methanol, 7% acetic acid)

Methanol	50.0	ml
Acetic acid	70.0	ml
H_2O (distilled water)	1000.0	ml

Method of gel staining for protein

A gel stained with 0.1 % Comassie blue R-250, 50% methanl, 10% acetic acid for 5-6 hrs. The gel was then washed in destian I for hr. with 1-2 changes and followed by destain II until the gel was cleared. After washing in distilled H_2O for a few times, the was dried in a gel air dryer (BIO-RAD)

5. SDS molecular weight markers (Sigma) consist of:

-Myosin, rabbit muscle	205	kDa
-β-Galactosidase, *Escherichia coli*	116	kDa
-Phosphorylase b, rabbit muscle	97	kDa
-Fructose-6-phosphate kinase, rabbit muscle	84	kDa
-Albumin, bovine serum	66	kDa
-Glutamic dehydrogenase, bovine liver	55	kDa
-Ovalbumin, chicken egg	45	kDa
-Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	36	kDa
-Carbonic anhydrase, bovine erythrocytes	29	kDa
-Trypsinogen, bovine pancreas	24	kDa
-Trypsin inhibitor, soybean	20	kDa
-α-Lactalbumin, bovine milk	14.2	kDa
-Aprotinin, bovine lung	6.5	kDa

6. Towbin transfer buffer pH 8.8 for Western blot analysis

(25 mM tris, 192 mM glycine, 20% methanol)

The buffer consisted of the wing ingredients:

Tris	3.03	g
Glycine	14.4	g
Methanol	200.0	ml
H_2O (distilled water) adjusted to	1000.0	ml

The buffer was pre-chill before use.

APPENDIX E

REAGENT FOR DETERMINATION OF ISOTYPE AND SUBISOTYPE OF MONOCLONAL ANTIBODIES USING ANTIBODY CAPTURED ON ANTI-Ig ANTIBODIES

Hybridoma sub-isotyping kit, mouse (Zymed) contents:

A) Rabbit anti-Mouse IgG1 (γ1 chain specific)

B) Rabbit anti-Mouse IgG2a (γ2a chain specific)

C) Rabbit anti-Mouse IgG2b (γ2b chain specific)

D) Rabbit anti-Mouse IgG3 (γ3 chain specific)

E) Rabbit anti-Mouse IgA (α chain specific)

F) Rabbit anti-Mouse IgM (μ chain specific)

G) Rabbit anti-Mouse kappa light chain

H) Rabbit anti-Mouse lambda light chain

I) Normal Rabbit Serum, (Negative Control)

J) Positive Control, Monoclonal Mouse IgG1
(Mouse IgG1 is in RPMI-1640 with 10 % FBS)

K) Substrate Buffer, Concentration (10X)
(1 M citrate, pH 4.2, containing 0.03 % H_2O_2)

L) ABTS Substrate, Concentrated (50X)
(2,2 – azino-di [3-ethylbenzthiazoline sulfonic acid])

M) Blocking Solution, Concentration (50X)
(25% BSA in PBS and 0.05% NaN_3)

N) HPR-Goat anti-Rabbit IgG (H+L), Concentrated (50X)

O) Goat anti-Mouse IgGAM, Concentrated (50X)
(0.5 mg/ml in PBS containing 10% glycerol and 0.05% NaN_3)

P) 50% Tween 20

APPENDIX F

BUFFRS AND SOLUTION FOR ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

1. 5% Blotto solution (Longyant et al., 1999)

Skimmed milk	5.0	g
PBS 0.15 M pH 7.2	100.0	ml
1% Merthiolate (Sigma)	1.0	ml
Triton X-100 (Sigma)	0.1	ml

2. Washing solution (0.5% Blotto)

5% Blotto solution (4.1)	50.0	ml
PBS 0.15 M pH 7.2	950.0	ml

3. 0.1 M Citrate Buffer pH 4.5

Sodium citrate	29.41	g
1% Merthiolate	10.0	ml
H_2O (distilled water) adjust volume to	1000.0	ml

The pH of the buffer was adjusted to 4.5 with 0.1 M HCl

4. 1 N H_2SO_4

Concentrated H_2SO_4	27.0	ml
H_2O (distilled water) adjusted volume to	1000.0	ml

5. O-Phenylenediamine dihydrochloride (OPD)

APPENDIX G

REAGENT AND SOLUTION FOR IMMUNOHISTOCHEMISTRY (IHC)

1. Coated slide solution

Gelatin	1.0	g
Clone alum (chromium potassium sulphate)	0.05	g
H_2O (distilled water) adjust volume to	100.0	ml

2. Davidson's fixative

95 % Ethanol	30.0	ml
100 % Formalin	20.0	ml
Glacial acetic acid	10.0	ml
H_2O (distilled water)	30.0	ml

3. Phosphate buffered saline (PBS) 0.15 M, pH 7.2

NaCl	8.0	g
KCl	0.20	g
KH_2PO_4	0.20	g
$Na_2HPO_4.7H_2O$	1.15	g
H_2O (distilled water) adjust volume to	1000.0	ml

4. Calf serum 10% (P_1^+)

Calf serum	10.0	ml
PBS	100.0	ml

5. Enrilich's acid hematoxylin

Hematoxylin	8.0	g
95% ethanol	400.0	ml
Aluminium potassium sulphate	8.0	g
Distilled water	400.0	ml
Glycerine	400.0	ml
Glacial acetic acid	400.0	ml

6. 0.2 % Eosin Y in 95% ethanol

Eosin Y	0.2	g
95% ethanol	100.0	ml

Methods of immunohistochemistry and indirect immunoperoxidase (Sithigorngul et al., 2000; 2002).

1. Immunnohistochemistry (IHC)

1.1 Cephalothoraces of Greenback Mullet (*Liza subviridis*) oocyte

1.2 Fixed in Davidson's fixative solution 24 h.

1.3 Washed in water 3 h.

1.4 Dehydrated in a graded ethanol series and N-butyl alcohol respectively

1.4.1 70% ethanol : one change at 3 h.

1.4.2 90% ethanol : one change at 3 h.

1.4.3 95% ethanol : two changes at overnight each.

1.4.4 95% ethanol : n-butyl alcohol (1:1) : one change at 1 h.

1.4.5 n-butyl alcohol : one change at 1 h.

1.4.6 n-butyl alcohol : xylene (1:1) : one change at 1 h.

1.4.7 Xylene : two changes at 1 h each.

1.4.8 Xylene : paraplast at 60°C (1:1) : one change at 30 min.

1.4.9 Paraplast at 60°C : three changes at 45 min each.

1.5 Infiltrated tissue by paraplast and embed in the block.

1.6 Cut tissue serial section 8 μm thickness as a ribbon by rotary microtome.

1.7 Transfer section into room temperature water, on the glass slide and transfer slide on the slide warmer 50°C and incubated slide at 50°C overnight to dry section.

1.8 Deparaffinized and rehydrated tissue sections through a xylene and a graded ethanol series.

1.8.1 Xylene : three change at 5 min each.

1.8.2 n-butyl alcohol : one change at 5 min.

1.8.3 95% ethanol : one change at 5 min.

1.8.4 90% ethanol : one change at 5 min.

1.8.5 80% ethanol : one change at 5 min.

1.8.6 70% ethanol : one change at 5 min.

1.8.7 Distilled water : one change at 5 min.

1.8.8 10% formalin : one change at 10 min.

1.8.9 Distilled water : five changes at 5 min each.

1.8.10 PBS : three changes at 5 min each.

1.9 Removed slides from slide basket, dry around tissue section with vacuum pump. Tissue must never dry out. Place each slide in a humidity chamber as it is prepared.

2. Indirect immunoperoxidase method

2.1 Blocking

2.1.1 Covered tissue sections with P_1^+ solution.

2.1.2 Incubated slides for 30 min in humidity chamber, room temperature.

2.2 First antibody

2.2.1 Removed P_1^+ solution from section.

2.2.2 Dropped first antibody cover each section

2.2.3 Incubated at 37°C for 5 h in humidity chamber.

2.2.4 Washed section with distilled water: one change rapidly.

2.2.5 Washed section with PBS: three changes at 10 min each.

2.3 Second antibody

2.3.1 Removed PBS from section.

2.3.2 Dropped second antibody covers each section (goat anti-mouse horseradish peroxidase (GAM-HRP) as second antibody.

2.3.3 Incubated at 37°C for 3 h in humidity chamber.

2.3.4 Washed section with distilled water : one change rapidly.

2.3.5 Washed section with PBS : three changes at 10 min each.

2.4 Peroxidase activity was revealed by incubation with 0.03% 3',3 diaminobenzidine tetrahydrochloride and 0.006% hydrogen peroxide in PBS at 5 min.

2.5 Washed section with water.

3. Counter-stained with eosin Y

3.1 Dehydrated in a graded ethanol series 70%, 80%, 90% and 95% : one change at 5 min each.

3.2 Counter-stained with eosin Y.

3.3 Dehydrated in n-butyl alcohol : one change at 5 min.

3.4 Dehydrated in n-butyl alcohol : xylene (1:1) : one change at 5 min.

3.5 Cleaned in xylene : three changes at 5 min each.

3.6 Mounted in permount as permanent slide.

BIOGRAPHY

NAME : Miss Pajongsuk Sutarut

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ACADEMIC PRESENTATION :

1. Sutarut, P., Puanglarp, N., Longyant , S., Sithigorngul, P., and Menasveta, P. Detection and characterization of vitellogenin and zona radiate proteins for the determination of xenoestrogen in water.31th Congress on Science and Technology of Thailand. 18-20 October 2005.Suranaree University of Technology of Thailand, Nakhon Ratchasima

2. Sutarut, P., Puanglarp, N., Longyant , S., Sithigorngul, P., and Menasveta, P. Production of monoclonal antibodies specific to vitellogenin and zona radiate protein of greenback mullet (*Liza subviridis*).32th Congress on Science and Technology of Thailand. 10-21 October 2006. Queen Sirikit National Convention Center, Bangkok, Thailand