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Maturation Induction of *Pangasius hypophthalmus* Using Gonadotropin Releasing Hormone Analogue (GnRHa) in Combination with Domperidone, in Oil Suspension Dosage Forms

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Abstract

This study has investigated the effects of gonadotropin releasing hormone analogue (GnRHa) in combination with domperidone (DOM) suspended in soybean oil injected both in male and female Pangasius Catfish (*Pangasius hypophthalmus*) and testosterone (T) or estradiol (E_2) oil suspension injected in male or female fish respectively on the improvement of gonad development. Twenty five males or females (1.5 years old, 0.9 kg body weight) were injected with the hormone samples. Before and after injection at 30,150, 240 and 300 days, E_2 and T levels in the blood samples were determined by electrochemiluminescence immunoassay. Gonadosomatic indices (GSI) and oocyte development at initial and 30,150, 240 and 300 days after injection were also determined for maturity examination. The highest E_2 levels in females and males were 1,423 and 106.8 $pgml^{-1}$ after 240 and 150 days, respectively of injecting with 300 $ugkg^{-1}$ GnRHa in combination with 20 $mgkg^{-1}$ of DOM. The highest T levels in females and males were 10.9 and 14.6 $ngml^{-1}$ after 240 and 150 days of injecting with 100 and 200 $ugkg^{-1}$ GnRHa in

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combination with 5 and 10 mgkg^{-1} of DOM respectively. The highest GSI and the more developed stages of oocytes and spermatocytes were observed after 150 and 240 days of the injection. This study demonstrated not only that the 1.5 years old *P. hypophthalmus* can be artificially induced for maturation after being injected with GnRHa in combination with DOM indicating the pre spawning in January - April, spawning in May - September, and post spawning in October - December, but also the obtained information can be used as a breeding model for the Mekong giant catfish (*Pangasianodon gigas,* Chevy), which is closely related to this fish.

Introduction

Pangasius catfish (*Pangasius hypophthalmus*), needs at least 3 years to be mature in captivity in earthen ponds. Hormone induction for early maturation is needed for this fish in mass production and economic aspects. Usually, exogenous hormones affecting ovulation have short duration. One injection may last for 6 to 12 hrs (Van der Kraak et al. 1983). Hence, two to four injections are needed. Frequent injection can cause fish to suffer from stress and may result in various problems for hatchery management. For example, repetitive handling of broodstock requires substantial labor, time and monitoring. Carp pituitary, which has been normally used to induce spawning is expensive. Gonadotropin releasing hormone analog (GnRHa, or Buserelin, Suprefact®) in combination with domperidone (DOM), an antagonist of dopamine which is produced when fish are stressed, has been frequently used (Peter et al. 1993). Buserelin is an analogue of GnRHa, which regulates gonadotropin hormone (GtH). GtH comprises of luteinizing hormone (LH) and follicle stimulating hormone (FSH) which can affect the development of ovary and testis. The essential hormones for ovulation, GnRH together with the GnRH receptor, which are located on the gonadotrope membrane in the pituitary gland, can stimulate gonadotropin production. Gonadotropin then will be released into the blood by G protein-coupled receptor systems (Chumpoothawip 1998). Testosterone is involved in spermatogenesis and is a precursor of estradiol, which produces vitellogenin for vitellogenesis during egg development (Scott et al. 1980). More than two consecutive GnRHa injections are necessary to give a slow final oocyte maturation in striped bass (Van der Kraak et al. 1983). Long-term GnRHa delivery systems are essential for the control of maturation of this fish. GnRH analogues are advantageous since they resist to enzymatic degradation when injected into fish, resulting in a more prolonged stimulation of gonadotropin hormone release, in comparison to the native GnRH peptide (Goren et al. 1990; Zohar et al. 1989 1990 1995a 1995b). Suitable concentrations of GnRHa in sustained release delivery systems have been shown to successfully stimulate gonad development, reduce stress caused by multiple handling, and assist the broodstock to become ready for maturation (Zohar and Mylonas. 2001). In this study, effects of injecting an artificial GnRH hormone, buserelin acetate, in combination with DOM or testosterone or estradiol alone suspended in soybean oil on sex hormone levels, gonad and germ cell development of *P. hypophthalmus*, a fish which is closely related to the giant catfish (*Pangasianodon gigas,* Chevy) reared in earthen ponds were investigated.

Materials and Methods

Fish samples

This study was conducted during April - September in 2001. Twenty five immature males and females *P. hypophthalmus* (a total of 50), 1.5 years old, with an average body weight of 0.9 kg, reared in earthen ponds (130 m^2) at the Department of Fisheries Technology, Maejo University, Chiang Mai, Thailand, were used. Sex identification was done by physical examination which was confirmed by biopsy of the gonad. Each fish was marked by injecting Alcian blue dye at the different parts of the fins. Feeds containing 20% protein (8.15% fishmeal, 4.9% soybean, 4% rice bran and 3% broken rice in combination with 2% premix and 1% tuna oil) were prepared and fed to the fish at the ratio of 2% body weight per day.

Hormone preparation

Suprefact® (10,000 $\mu g \cdot ml^{-1}$ of buserelin acetate, a GnRH analogue, Hoechst AG, Germany) was evaporated and centrifuge under reduced pressure (Centrivap Model LABCONCO 78120-01, USA) at 30 ± 2°C, 2,500 rpm for 3 hr. Motilium® tablet (10 mg per tablet of DOM) was ground, dispersed in ethanol and then filtered. The filtrate was evaporated at 45°C using a rotary evaporator (Rotavapor R-124 Büchi, Switzerland). The contents of buserelin and DOM in the dried powder were analyzed by HPLC (Thermo Separation Products, USA UV 1000/P2000). The calculated amounts of buserelin and DOM were dispersed aseptically in sterile soybean oil, and kept at 4°C until use. The chemical structural and molecular weight of buserelin acetate, was presented in figure 1 (British Pharmacopoeia 1998).

Hormone treatment

For the GnRHa treatment, twenty five males and females were used. Fish were divided into 4 groups (T1-T4). Each group contained 5 males and 5 females. For estradiol (E_2) or testosterone (T) treatment, five males and

5-Oxo-Pro-His-Trp-Ser-Tyr-D-Ser (t-Bu)-Leu-Arg-Pro-NHEt

$C_{60}H_{86}N_{16}O_{13}$ **MW = 1239.5**

Fig. 1. Chemical structure of buserelin, an analogue of the natural gonadotropin-releasing hormone (GnRH)

five females fish were also used. They were divided into two groups (T5 and T6). Each group contained either 5 females or 5 males. Each fish was intramuscularly injected with the hormone samples of 1 $mlkg^{-1}$ (15 January 2001). The treatment program is shown in table 1.

Testosterone and estradiol analysis

Half milliliter of blood sample was drawn from the caudal vein of each fish before and after 30, 150, 240 and 300 days of injection. The blood sample was centrifuged at 10,000 rpm for 5 minutes. From 50 microliters of the serum, E_2 and T levels were determined by a polyclonal antibody from rabbit and a monoclonal from mouse antibody specifically directed against E_2 and T, respectively and measured by an electrochemiluminescence immunoassay analyzer (Elecsys1010, Roche Germany).

Gonadosomatic index (GSI)

One male and one female fish from each treatment before and 30,150, 240 and 300 days after injection were sacrified and the gonadosomatic indices (GSI) of ovary or testis were calculated according to the following equation (Nikolsky 1963):

$$GSI = \frac{\text{gonad weight (kg)} \times 100}{\text{bodyweight (kg)}}$$

Gonad histology

Ovary or testis samples taken from one female or male fish before and after 30, 150, 240 and 300 days of injection from each treatment were fixed in 10% formalin, hydrated and embedded in paraffin. The 6 mm thick sections were stained with haematoxylin and eosin. The stages of oocytes or spermatocytes from the middle parts of the ovary or testis were examined under a microscope (Nikon SE, Japan) at the magnification of 100X and 400X and the four stages were investigated according to the previous study (Jarimopas et al. 1994 and Prat et al. 2001). Percentages of oocytes at different stage, were presented, whereas spermatocyte stages were photographed.

Data and statistical analysis

E_2 and T levels were presented as mean ± standard error. One way analysis of variance (ANOVA, $P<0.05$) and a mean different test of E_2 and T levels were analyzed by a Statistic Package for Social Science (SPSS) at the significant level of $^{*}P<0.05$. Comparison of the treatment mean difference was analyzed by the Least Significant Test (LSD).

Results

Testosterone (T) and estradiol (E_2) levels

Before hormone injection in 15 January 2001 of all treatments, T and E_2 levels in males and females were less than 0.02 $ngml^{-1}$ and 10 $pgml^{-1}$, respectively. After 30 days (15 February 2001) of hormone injection, the one and a half year old fish that received GnRHa at 300 $mgkg^{-1}$ in combination with DOM 20 $mgkg^{-1}$ or E_2 at 20 $mgkg^{-1}$ in soybean oil showed the highest level at 0.12 $ngml^{-1}$of T in males and at 142 $pgml^{-1}$of E_2 in females. T and E_2 levels in males and females injected with GnRHa at 200 and 300 $mgkg^{-1}$ in combination with DOM at 10 or 20 $mgkg^{-1}$, respectively after 150 days (15 June 2001) were higher than after 30 days (15 February 2001). These levels were significantly different from those before injection. The highest values of T and E_2 were at 14 $ngml^{-1}$ in males and 581 $pgml^{-1}$ in females injected with GnRHa at 200 $mgkg^{-1}$ in combination with 10 $mgkg^{-1}$ of DOM after 150 days (15 June 2001), which was significantly different from before injection. At 240 days (15 September 2001) after the injection, the highest values of T and E_2 were at 14 $ngml^{-1}$ in male injected with 200 $ugkg^{-1}$ of GnRHa in combination with DOM at 10 $mgkg^{-1}$, and 1,423 $pgml^{-1}$ in females injected with 300 $mgkg^{-1}$ of GnRHa in combination with DOM at 15 $mgkg^{-1}$, respectively. These values were also significantly different from before injection. T and E_2 levels decreased after 300 days (15 November 2001) of hormone injection (Table 2).

GSI and reproductive parameters

After 30 days (15 February 2001) of hormone injection, GSI values of all treated groups were increased in comparison to the control group. After 150 days of hormone injection, GSI increased in all treatments. The highest GSI value was 16.52 in females and 6.54 in males which received 200 $mgkg^{-1}$ of GnRHa in combination with 10 $mgkg^{-1}$ of DOM. After 240 days (15 September 2001) of hormone injection, GSI was 24.26 and 9.73 in females and

Table 1. The treatment program of *P. hypophthalmus* (aged 1.5 year old) injected with GnRHa in combination with domperidone (DOM) or Testosterone (T) or Estradiol (E_2) in soybean oil suspension dosage forms

Treatment No.	Sex	Number	Average body Weight (kg)	Description of the sample	Amount injected ($mlkg^{-1}$)
T1	Female	5	0.7±0.22	soybean oil only	0.7
	Male	5	0.5±0.25		0.5
T2	Female	5	0.7±0.27	100 $mgkg^{-1}$ GnRHa in combination	0.7
	Male	5	0.8±0.30	with 5 $mgkg^{-1}$ DOM in soybean oil	0.8
T3	Female	5	0.7±0.32	200 $mgkg^{-1}$ GnRHa in combination	0.7
	Male	5	0.6±0.19	with 20 $mgkg^{-1}$ DOM in soybean oil	0.6
T4	Female	5	0.5±0.16	300 $mgkg^{-1}$ GnRHa in combination	0.6
	Male	5	0.6±0.27	with 20 $mgkg^{-1}$ DOM in soybean oil	0.5
T5	Female	5	0.4±0.32	20 $mgkg^{-1}$ estradiol (E_2) in soybean oil	0.8
T6	Male	5	0.8±0.23	20 $mgkg^{-1}$ testosterone (T) in soybean oil	0.4

Table 2. Serum concentrations of testosterone (T) and estradiol (E_2) in *P. hypophthalmus* at 0, 30, 150, 240 and 300 days of hormone injection in treatment T1 to T6

Treatment	Sex	Hormonal level at days after injection									
		0 day (January 2001)		30 days (February 2001)		150 days (June 2001)		240 days (September 2001)		300 days (November 2001)	
		T (ng/ml)	E_2 (pg/ml)	T (ng/ml)	E_2 (pg/ml)	T (ng/ml)	E_2 (pg/ml)	T (ng/ml)	E_2 (pg/ml)	T (ng/ml)	E_2 (pg/ml)
T1 (Soybean oil)	Female	0.02±0.01	10±1.41	0.02±0.01	56.1±28.44	1..9±0.69	234±39.17	5.3±1.31	725.8±36.06	0.45±0.17	341±24.63
	Male	0.02±0.01	10±1.40	0.02±0.01	42.3±16.88	6.3±1.49	45.9±10.78	10.5±3.02	35.1±5.80	0.21±0.05	37±9.82
T2 (GnRHa 100 mg/kg	Female	0.02±0.01	10±1.70	0.02±0.01	25.4±9.05	0.8±0.36	298.9±18.43	10.9±3.48	611±50.77	1.5±0.23	554±45.43
+ DOM 5 mg/kg)	Male	0.02±0.01	10±1.76	0.02±0.01	74.6±10.91	7.4±2.59	13.1±3.52	13.9±2.61	36.4±4.32	0.26±0.14	-
T3 (GnRHa 200 mg/kg	Female	0.02±0.01	10±1.60	0.02±0.01	-	3.3±1.55	549.3±31.30	9.4±2.06	903.4±26.15	0.2±0.09	207±39.70
+DOM 10 mg/kg)	Male	0.02±0.01	10±1.41	0.02±0.01	26.9±6.48	14.6±4.03	91.5±6.83	14±3.64	43.2±6.68	2.8±0.49	122±15.13
T4 (GnRHa 300 mg/kg	Female	0.02±0.01	10±1.90	0.02±0.01	12.4±3.35	6.1±4.09	581.8±23.51	6.4±1.69	1,423±55.45	0.02±0.01	909±80.83
+DOM 20 mg/kg)	Male	0.13±0.01	10±1.60	0.128±0.01	10±5.46	9.2±4.29	106.8±9.87	8.6±2.68	30.6±4.88	0.21±0.08	33.8±7.08

Note: GnRHa = gonadotropin releasing hormone analogue, DOM = domperidone, E_2= estradiol, T = testosterone

males, respectively. GSI dropped in all treatments at 300 days (15 November 2001) after hormone injection (Table 3).

Figure 2 showed that the stages of oocytes after 150 days (15 June 2001) of injecting with soybean oil and GnRHa in combination with DOM were well developed with the maximum maturation stage of about 44%. After hormone injection, oocytes developed in the same trend as GSI values. Figure 3 showed the more developed spermatocytes of fish treated with hormone than that treated with soy bean oil only.

Discussion

During 150 days (15 June 2001) to 240 days (15 September 2001) after hormone injection, T and E_2 levels still increased, but decreased after 300 days of hormone injection (15 November 2001). This may be due to the injection of soybean oil and hormone which integrated with the period of the reproductive cycle of this tropical fish, i.e. pre spawning in February, spawning in June to September and post spawning in November. E_2 levels in males and females which received GnRHa at 300 $mgkg^{-1}$ in combination with DOM at 15 $mgkg^{-1}$ after 150 and 240 days of the injection were higher than those treated with only soybean oil ($P< 0.05$). This agreed with the previous work that GnRHa alone or in combination with DOM induced a sharp increase in plasma E_2 concentration of Sea Bass (*Dicentrachus labrax)* (Prat et al. 1999). After administration of GnRHa, higher concentration of E_2 was found in vitellogenic females than in postvitellogenic females (Sower et al. 1984). During our study period, T and E_2 levels of *P. hypophthalmus* gave almost the same trend, but the T level decreased earlier than the E_2 level. This has also been demonstrated in Sea Bass (Prat et al. 2001). The captive catfish (*Clarias macrocephalus*) was found to have a group synchronous pattern of ovarian development during the annual reproductive season, owing to the presence of oocytes in all stages of the development throughout the annual cycle. The highest GSI of this fish found in July - September, was ranging from 17 to 20, while T levels were ranging from 50 to 55 $ngml^{-1}$. The E_2 levels were ranging from 10 to 15 $ngml^{-1}$. Changes in various reproductive parameters and steroid hormone levels have indicated that January - March, April to June, July to September and October - December correspond to the refractory, preparatory, spawning and post spawning periods, respectively, (Tan-Fermin et al. 1997).

In our study, GSI values of *P. hypophthalmus* (1.5 year old) during February - November in the treated group injected with GnRHa in combination with DOM tended to be higher than the control group injected with soybean oil only or the treated group injected with T or E_2 suspended in soy bean oil. GSI values in June and September (spawning season) were higher than in February and November (pre and post spawning season). This may have resulted from the annual reproductive cycle as well as the injection of GnRHa. Moreover, the oocyte development stage appeared to be more in June and September than in February and November.

Table 3. GSI values of *P. hypophthalmus* after 30, 150, 240 and 300 days of hormone injection in treatment T1 to T6

Treatment	Sex	Weight (g)	GSI values at day after injection			
			30 (15 February 2001)	150 (15 June 2001)	240 (15 September 2001)	300 (15 November 2001)
T1 (Soybean oil)	Female	982	0.27	13.94	18.11	2.4
	M ale	1,090	0.07	6.54	7.5	0.17
T2 (GnRHa 100 mg·kg^{-1} + DOM 5 mg·kg^{-1})	Female	1,046	0.43	14.5	24.26	3.56
	Male	968	0.05	4.62	7.84	0.13
T3 (GnRHa 200 mg·kg^{-1} +DOM 10 mg·kg^{-1})	Female	1,048	0.38	16.52	19.65	3.78
	Male	996	0.32	6.34	9.73	0.17
T4 (GnRHa 300 mg·kg^{-1} +DOM 200 mg·kg^{-1})	Female	1,158	0.55	10.9	19.18	2.61
	Male	1,115	0.12	5.98	8.50	0.15
T5 (E2 20 mg·kg^{-1})	Female	695	0.45	8.42	-	-
T6 (T 20 mg·kg^{-1})	Male	869	0.15	5.14	-	-

Note: GnRHa = gonadotropin releasing hormone analogue, DOM = domperidone, E_2 = estradiol, T = testosterone

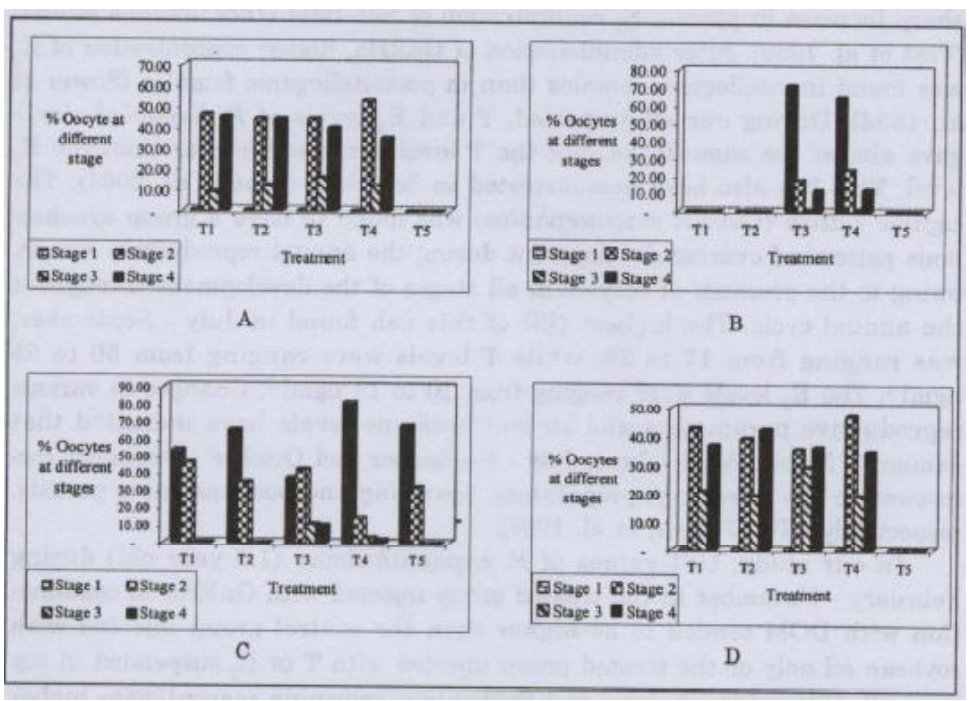

Fig. 2. Percentages of the different stages of oocytes of *P. hypophthalmus* (1.5 year old) at different stage in each treatment A=30, B=150, C=240 and D=300 days after hormone injection.
T1 Soybean oil; T2 GnRHa 100 mgkg^{-1} in combination with DOM 5 mgkg^{-1}; T3 GnRHa 200 mgkg^{-1} in combination with DOM 10 mgkg^{-1}; T4 GnRHa 300 mgkg^{-1} in combination with DOM 20 mgkg^{-1}; T5 E_2 20 mgkg^{-1}; Stage 1 Central germinal vesicle; Stage 2 Migrating germinal vesicle; Stage 3 Peripheral germinal vesicle; Stage 4 Maturation (germinal vesicle breakdown)

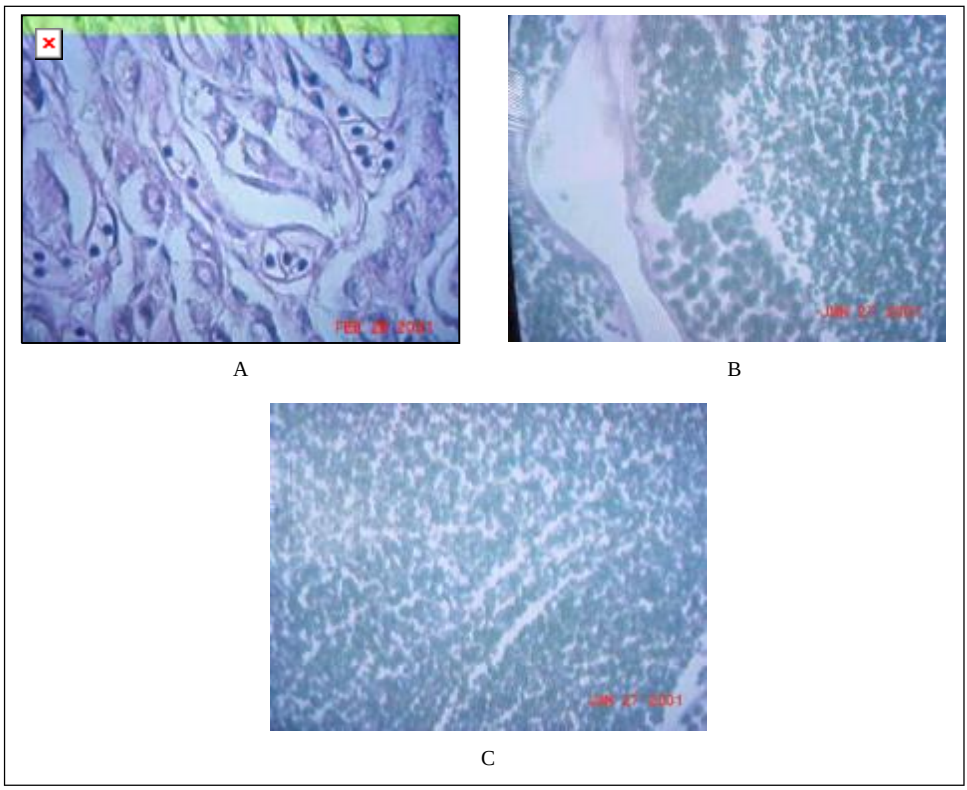

Fig. 3. Spermatocyte development of *P. hypophthalmus* after injecting with soybean oil and the hormone suspended in soybean oil
a) Spermatocyte 30 days (February 2001) after treated with soybean oil
b) Spermatocyte and spermatozoa 150 days (June 2001) after treated with soybean oil
c) Spermatid and spermatozoa 150 days (June 2001) after treated with GnRHa 200 mg/kg in combination with DOM 10 mg/kg

One and a half years old *P. hypophthalmus*, both males and females in our study reached maturation after 187 days of hormone injection. The female eggs were obtained more successfully from fish treated with GnRHa in combination with DOM than the other groups. However, fertilization was also observed in the control group injected with soybean oil only (data not shon). Soybean oil is the oil derived from plants and usually contains stigmasterol, a cyclopentanoperhydrophenanthrene skeleton compound which is one of the precursors for sex steroidal hormone production (Dence 1980).

Conclusion

One and a half year old *P. hypophthalmus* (0.9 kg body weight) after 150 and 240 days injected with GnRHa at 100 to 300 $mgkg^{-1}$ in combination with DOM at 5 to 20 $mgkg^{-1}$ had the higher T and E_2 levels indicating more

sexual development than fish before injection and the control fish injected with soybean oil only. Also, their GSI, oocyte and spermatocyte development seemed to be better than those treated with T or E_2 or the control group. In addition, these reproductive parameters were related to the annual reproductive cycle i.e., June and September (spawning season), February and November (pre and post spawning season). This study has demonstrated that *P. hypophthalmus* could reach maturation at 1.5 years old of age by injecting with hormone suspended in soybean oil. The information obtained from this fish will be beneficial as a model for the planning of artificial propagation of its closely related brooder, the Mekong giant catfish reared in ponds by hormone manipulation and broodstock management in order to relieve the pressure on the diminishing number of this wild giant catfish, and to develop the techniques for fish cultures.

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