Evaluation of the effectiveness and cost of different hormones in stimulating the spawning of thin lipped grey mullet, *Liza ramada*

Original Article

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ABSTRACT

Introduction: It is known that mullets are not fully mature in captivity and therefore do not lay eggs. Therefore, hormonal preparations should be used to stimulate spawning.

Aim of the work: To evaluate the effectiveness and cost of using different types of hormones; pituitary hormone of carp, human chorionic gonadotropin, gonadotropin - releasing hormones in stimulating spawning of *Liza ramada*.

Materials and Methods: In this study, the pituitary gland was selected for two types of carp; common carp, silver carp, and human chorionic gonadotropin, as well as buserelin acetate and triptorelin acetate to stimulate the spawning of *Liza ramada*. In this regard, these hormones were tested on the rate of ovulation, fertilization rate, embryonic development and hatching rate, as well as an economic evaluation of the cost of injecting these hormones.

Results: The results showed the effectiveness of the used hormones to stimulate ovulation, fertilization and hatching, but at different rates. The use of the pituitary gland of silver carp induced a higher ovulation rate than those of common carp and human chorionic gonadotropin. Buserelin acetate induced higher ovulation, fertilization and hatching rates than those of triptorelin acetate. From the economic point of view, the use of the pituitary gland of silver carp as a stimulant dose and also the use of buserelin acetate as a resolving dose is less expensive compared to the use of other tested hormones.

Conclusion: The use of the pituitary gland of silver carp and buserelin acetate was effective to stimulate the spawning of mullets and less expensive.

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Key Words: Economic efficiency, hormone, *Liza ramada,* spawning.

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INTRODUCTION

Captive propagation of fishes is essential to meet the growing demands placed on finite fisheries resources. To this end, the aquaculture industry must find reliable and cost effective methods to spawn and culture species of interest. The reproduction of many fish species in hatcheries is impossible without the application of hormonal preparations[1-3]. Adult fish, especially the females, are generally unable to produce their gametes naturally under hatchery conditions. The most cost- and time-effective way to obtain ovulated eggs and sperm is stimulating the fish with hormones. Reproductive hormones have been used to stimulate reproductive processes and induce ovulation, spermiation and spawning. Among various reproductive hormones, pituitary gland (PG) or human chorionic gonadotropin (HCG) and gonadotropin releasing hormone (GnRHa) are commonly used for inducing gametogenesis and spawning in many fish species[1-3]. Further research is needed; in particular, optimal application protocols need to be determined for each species individually.

The mullets are euryhaline species spawning only in salty water but can also grow in brackish and fresh waters. Culture of mullets restricted to areas of Egypt where seed could be collected from the wild and distributed. Its production is still remaining restricted until the induced spawning techniques are developed. However mullets, in captivity, do not spawn naturally and show some degree of reproductive dysfunction[4-6]. The inability of fish in captivity to complete its reproductive cycle is most likely due to a failure at one or more sites along the hypothalamo-hypophyseal-gonadal axis[7]. The failure of captive mullet fish to undergo final oocyte maturation (FOM) is thought to be caused by the shortage of gonadotropin synthesis[5] and/or the lack of pituitary gonadotropin (GTH) release[8] at the end of vitellogenesis process. Under natural conditions, dopamine inhibits the action of GnRH and the release of GTHs through a feedback mechanism. Removing inhibitory effect of dopamine by antidopamines such as metoclopramide (MET) resulted in oocyte maturation and ovulation of grey mullet, implying the serious inhibitory effects of dopamines in the fish[9].

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Generally for mass fry production in finfish, the use of GnRH analogue in combination with dopamine antagonist (DA) was recommended for spawning induction[10]. Accordingly, co-treatment of carp pituitary extract or human chorionic gonadotropin and different analogs of GnRHa in combination with DA were adapted to induce spawning in fish[3, 11-15].

Lack of knowledge on the effectiveness and cost of using different types of hormones; pituitary hormone of carp, human chorionic gonadotropin, gonadotropin-releasing hormones combined with a dopamine antagonist in stimulating spawning of thin-lipped grey mullet (*L. ramada*). Therefore, the present study aimed to evaluate the efficacy and cost of the different injection protocols for stimulating oocyte final maturation, ovulation, spermiation and spawning in ripe spawners of thin-lipped grey mullet, reared in freshwater fish farms.

MATERIALS AND METHODS

Study Site:

This study was carried out at both of El-Serw Fish Research Farm and El-Matareyya Research Station in the period between 1st. January and 31st. December, 2017.

Hormones and chemicals

The hormones employed were:

• Carp pituitary glands (CPG): acetone - dried common carp (Cyprinus carpio) and silver carp (Hypophthalmichthys molitrix) pituitary glands which obtained from sexually mature donor fish.

• Human chorionic gonadotropin (HCG) "pregnyl" (Nile Co. for Pharmaceuticals, Cairo, ARE)

• Luteinizing hormone-releasing hormone agonist analogue (LH-RHa): Buserelin acetate (Glp-His-Trp-Ser-Tyr-Ser-tBu-Leu-Arg-Pro-NHEt) (Sigma, Steinheim, Hamburg, Seelze, Germany).

• Luteinizing hormone - releasing hormone agonist analogue (LH-RHa): Triptorelin Acetate (Pyr-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2 acetate salt) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA)

• Dopamine antagonist: The water-soluble dopamine receptor antagonist: metoclopramide (Sigma, Steinheim, Hamburg, Seelze, Germany).

Induction of spawning

Seawater acclimation:

The present experiments were carried out during the natural spawning season of thin-lipped grey mullet. Mature fish of thin-lipped grey mullet obtained from El-Serw Fish Research Station (freshwater habitat). Based on spermiation of males and slightly distended abdominal condition of females sexes were identified. Mature breeders, at least two-years-old, with total weights (600-750) g and total lengths (30- 40) cm, were collected alive, by draining water completely, during the spawning season (November to January) from culture ponds. There were 70 females and 140 males selected from the captured fish used in the spawning induction experiments.

The fish were anesthetized in a solution (40 mg/L) of clove oil (Sigma) before handling[16]. Mature females of thin-lipped grey mullet were selected on the basis of the presence of a soft, swollen abdomen and protruding genital papillae. The maturity and the oocyte diameters of the females were staged by obtaining in vivo biopsy of the ovary using a polyethylene cannula[17]. The females that were used possessed oocytes whose diameters were greater than 600 µm. The diameter of at least 25 of the largest oocytes was recorded from each fish, and the position of the germinal vesicle (GV) was determined after clearing the cytoplasm for 10 min with a 1:1:1 v/v methanol:ethanol:acetic acid solution[18]. Ripe males, in which milt could be easily extruded, by gentle pressure on their bellies, were used. Selected breeders were acclimated in 2000-litre circular fiberglass tanks (10 fish/tank). In brief, fish were transferred to water 10% salinity (for 12 h) which gradually increased to 35% (for another 12 h).

The experimental design and the protocol of hormonal injection:

Acclimated breeders were transferred to 500-litre fiberglass tanks equipped with constant running ozonated seawater (35%) and aeration (Female + 2 males/tank) for induction of spawning. The hormones were injected, at 1 ml/Kg, into the dorsal musculature of fish adjacent to the dorsal fin. The used doses were calculated empirically depending on a series of preliminary experiments determining the optimal dose. The males of each spawner were injected with the same doses of female. The experimental design and the protocol of hormonal injection[3] as described below. Seven different injection protocols were used (10 females for each group):

Group 1: priming injection at a dose of 20,000 IU HCG/kg body weight followed, 24 h later, by a resolving injection of 100 µg triptorelin acetate + 5 mg Metoclopramide/kg body weight.

Group 2: priming injection at a dose of 20,000 IU HCG/kg body weight followed, 24 h later, by a resolving injection of 4 µg buserelin acetate + 5 mg Metoclopramide/kg body weight.

Group 3: priming injection at a dose of 20 mg common CPG/kg body weight followed, 24 h later, by a resolving injection of 100 µg triptorelin acetate + 5 mg Metoclopramide/kg body weight.

Group 4: priming injection at a dose of 20 mg common CPG/kg body weight followed, 24 h later, by a resolving injection of 4 µg buserelin acetate + 5 mg Metoclopramide/kg body weight.

Group 5: priming injection at a dose of 20 mg silver CPG/kg body weight followed, 24 h later, by a resolving injection of 100 µg triptorelin acetate + 5 mg Metoclopramide/kg body weight.

Group 6: priming injection at a dose of 20 mg silver CPG/kg body weight followed, 24 h later, by a resolving injection of 4 µg buserelin acetate + 5 mg Metoclopramide/ kg body weight.

Group 7: The control group of fish received saline solution (0.9% NaCl) only, without hormone addition.

Tissue processing and histology:

The fishes were anesthetized in a solution (20 mg/L) of clove oil (Sigma) before handling[16] and then perfused via the ascending aorta with 20 ml of normal saline, followed by 50 ml of Bouin's fluid at 4°C. The gonads were removed and post fixed in Bouin's fluid for 24 h at 4°C. The fixed gonads were thereafter dehydrated through graded ethanol solution, cleared and embedded in paraplast (M.P: 56–58 °C). Consecutive transverse sections of the gonads (4µm thickness) were stained with Harris's alum hematoxylin[19] and aqueous solution of eosin as a counter stain.

The response of ripe spawners and hormone cost during the induction of spawning:

Hormone treatments were evaluated using the following criteria: (i) egg production, (ii) number of fertilized eggs and (iii) number of hatched larvae. The eggs were collected from each spawning tank (10 tanks) approximately two hours after the estimated time of spawning. Eggs were collected with PVC (polyvinyl chloride) pipes and siphoned into a plastic circular egg-collection basket (10 litres) with nylon bottom (mesh size 200 µm). The volume of total eggs was estimated by cylinder. Number of ovulated eggs produced by each female was estimated by eggs ml^{-1} egg volume multiplying by total eggs volume, and then the number of eggs g^{-1} body weight was observed for each female. Three replicates of 500 eggs from each fish were randomly taken and used to estimate number of fertilized eggs and number of hatched larvae. Fertilization was determined under a dissecting microscope approximately 1 h after spawning when eggs were at the cleavage stage. The hormone cost, per each kilogram of spawner body weight, was determined for each hormonal protocol injection. Also, the hormone cost, per each produced 100000 fertilized eggs, was calculated.

Statistical analysis:

Differences between treatments were tested by one-way ANOVA using the treatment as factor of variance. Statistical significance was accepted at $P<0.05$.

RESULTS

L. ramada females attained to prespawning stage in freshwater ponds. Furthermore, the fresh water was suitable for full maturation of *L. ramada* males; which attained to ripe stage in freshwater ponds. The oocytes of prespawning females were in vitellogenic stage (tertiary yolk stage) with a centrally located germinal vesicle (GV) (Figs. 1 and 2).

Induced spawning and response of L. ramada to different injection protocols:

Freshwater *L. ramada* breeders were successfully acclimated to seawater (35%) prior to hormonal injection. As described in table (1), seven different injection protocols were used to stimulate oocyte final maturation, ovulation, spermiation and spawning in ripe spawners of thin-lipped grey mullet. The body weight of injected breeders was ranged from 640±40 to 670±60 gm.

All injected breeders (groups; 2-6) were spawned (43- 46) h after the first injection (Table 1). But the breeders of group 1 injected protocol (injected with pregnyl; HCG as priming injection at a dose of 20,000 IU/kg body weight followed, 24 h later, by resolving injection of 100 µg triptorelin acetate + 5 mg Metoclopramide/ kg body weight), gave a spawning rate of 90% after 48 h from the first injection as illustrated in table (1). However, the breeders of group 7 (control) was not spawned.

The ovulated eggs were free, pelagic and transparent and vary in diameter between 0.9 and 1.0 mm (Fig. 3). The fertilized eggs have more than one oil globule, depending on the type and the dose of hormone; first cleavage begins at approximately 40 min (Fig. 4). The blastodisc was situated on the bottom side of the floating egg (Figs. 3 and 4). Forty-four hours after the spawning, lens fully formed; pigmentation gets dark and various colour combinations noticed (Fig. 5). The embryonic development was completed and hatching occurred at 48:00 h after spawning. The body of a newly hatched larva remained curved for several hours after hatching. The larva had a large oval-shaped yolk sac with an oil droplet at its posterior end. The mouth and anus were not yet open. No pigmentation was recognized over the yolk (Fig. 6).

In general, the use of carp pituitaries as a priming injection at a dose of 20 mg/ kg body weight gave a higher rate of ovulation than using of pregnyl. Furthermore, the pituitary glands of silver carp were more effective in inducing oocyte final maturation, ovulation, spermiation and spawning; since the number of ovulated eggs was significantly higher than those produced by using pregnyl and common carp pituitary glands as shown in table (1). The addition of 4 µg buserelin acetate + 5 mg Metoclopramide/kg body weight as a resolving injection, in groups 2, 4 and 6, significantly increased the number of ovulated eggs (113±17; 155±12 and 169±21 respectively) in comparison with the use of 100 µg triptorelin acetate + 5 mg Metoclopramide/kg body weight in groups 1, 3 and 5 (105±13; 143±14 and 159±19, respectively) as indicated in table (1).

The number of fertilized eggs followed the same

manner as the number of ovulated eggs in the different injection protocols; since the use of carp pituitary glands as a priming dose followed by buserelin acetate gave a higher values (29±5, 46±5 and 54±5 for groups 2, 4 and 6, respectively) than those used the pregnyl followed by triptorelin acetate (25±3; 40±6 and 47±6 for groups 1, 3 and 5 respectively). Similarly, the number of hatched larvae in buserelin acetate groups (15±4; 23±4 and 28±6 for groups 2, 4 and 6 respectively) was significantly higher than those in triptorelin acetate groups (12±3; 20±3 and 24±5 for groups 1, 3 and 5 respectively) as shown in table (1).

The use of carp pituitary glands as a priming dose followed by buserelin acetate as a resolving injection minimizes the cost of hormone injection (Egyptian Pound)/ kg body weight and consequently the production cost of 100000 fertilized eggs (table 1).

Table 1: The response of *L. ramada* ripe spawners to hormonal treatment and hormone cost during the induction of spawning

Spawned group	Body weight	Response of ripe spawners to hormonal treatment					Hormone cost	
		Spawning rate (%)	Time of spawning after 1st injection (h: min)	Number of ovulated eggs (x g^{-1} fish)	Number of fertilized eggs (x g^{-1} fish)	Number of hatched larvae (x g^{-1} fish)	Hormone cost/Kg body weight (Egyptian Pound)	Hormone cost per 100000 of fertilized eggs (Egyptian Pound)
Group 1	650±36	90[a]	48:00[a]	105±13[a]	25±3[a]	12±3[a]	600[a]	2400[a]
Group 2	640±40	100	46:30[b]	113±17[b]	29±5[b]	15±4[b]	520[b]	1793[b]
Group 3	660±52	100	46:00[b]	143±14[c]	40±6[c]	20±3[c]	650[c]	1625[c]
Group 4	646±38	100	45:00[c]	155±12[d]	46±5[d]	23±4[d]	570[d]	1239[d]
Group 5	655±45	100	44:00[c]	159±19[d]	47±6[d]	24±5[d]	550[d]	1170[d]
Group 6	657±55	100	43:15[d]	169±21[e]	54±5[e]	28±6[e]	470[e]	870[e]
Group 7 (Control)	670±60	No spawning	-	-	-	-	-	-

Data are reported as means ± SD.
Significantly different means ($P < 0.05$) are indicated by different letters (Tukey's test).

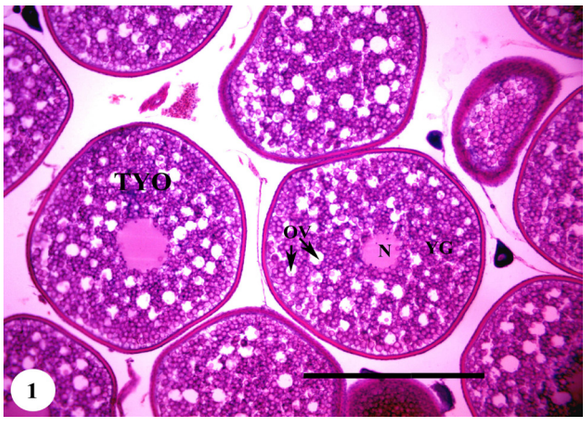

Fig. 1: Transverse sections of ovaries of *L. ramada*, stained with Harris's hematoxylin and eosin, showing ovary of post-vitellogenic female before injection of hormones at tertiary yolk oocyte (TYO) which has central-located nucleus (N), ooplasm impregnated with yolk globules (YG) and lesser number of oil vesicles (OV) distributed in the ooplasm. Scale bar = 500 µm.

Fig. 2: Macroscopic view of prespawning tertiary yolk oocytes which have central-located nucleus (N), ooplasm impregnated with yolk globules (YG). Scale bar = 500 µm.

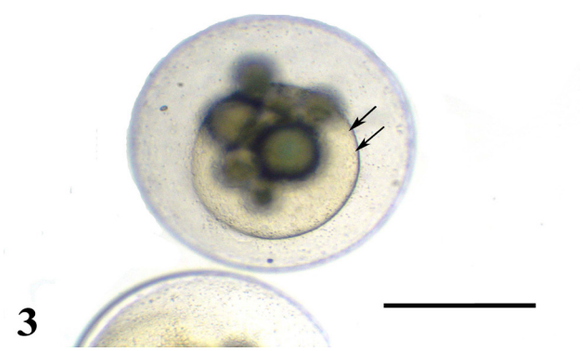

Fig. 3: Macroscopic view of ovulated eggs with blastodisc situated on the bottom side of the floating egg (arrows). Scale bar = 500 µm.

Fig. 4: Macroscopic view of fertilized eggs at Two-cell stage (arrows). Scale bar = 500 µm.

Fig. 5: Macroscopic view of developed embryo at 44 h post fertilization; lens fully formed; pigmentation gets dark and various colour combinations noticed. Scale bar = 500 µm.

Fig. 6: Macroscopic view of hatched larvae exhibiting a large oval-shaped yolk sac with an oil droplet at its posterior end. The mouth and anus were not yet open. Scale bar = 1000 µm.

DISCUSSION

In the present study, injection of pituitary extracts and HCG in combination with LH-RHa and dopamine antagonist (metoclopramide) induced the final stages of maturation, ovulation and spawning in *L. ramada*. Understanding the coordination between environmental factors and the fish internal regulating factors is very important for successful reproduction of fish. Mullets do not spawn spontaneously when reared in captivity. Failure of captive mullets to undergo FOM, without hormonal injection, is thought to be caused by the shortage of gonadotropin synthesis[4-6]. GnRHa and HCG act at different endocrine levels to induce oocyte maturation and ovulation, and this may explain the different results observed[20-22]. Both gonadal and extragonadal hormones are rather equally essential for the induction of circadian ovarian cycle of the grey mullet[23]. Exogenous GTH preparations acts directly at the level of the gonad, and LH-RHa release the endogenous GTH stores from pituitary. Endogenous GTH, in turn, acts at the level of the gonad to induce steroidogenesis and the process of ovulation and spermiation. In this work, we used pituitary extracts (20 mg/ kg body weight) or HCG (20000 IU/ kg body weight) as priming injection. Alike, previous investigators used HCG and pituitary glands successfully for the induction of ovulation and spawning in a number of fishes[24-31].

A large number of synthetic analogues of the native peptide were prepared to develop both long-acting super-active analogues (agonists) and inhibitory analogues (antagonists). The classic action of GnRH-I agonists that have been chemically synthesized is to augment efficacy (half-life), potency and binding to the receptor were compared to native GnRH-I[32] whereas two types of super-active agonists revealed. The first was decapeptides (GnRHa-I) like triptorelin, and the second was nonapeptides (GnRHa-II) like buserelin[33]. Later, GnRH-I has become the major stimulator of gonadotropins and probably other pituitary hormones as well, whereas GnRH-II functioned as neuromodulators, affecting reproductive behavior[34]. In the present study, the pituitary gland for two types of carp; common carp, silver carp, and human chorionic gonadotropin, as well as buserelin acetate and triptorelin acetate was selected to stimulate the spawning of *Liza ramada*. The results showed the effectiveness of the used hormones to stimulate ovulation, fertilization and hatching, but at different rates. The use of the pituitary gland of silver carp has given higher ovulation rate than those of common carp and the human chorionic gonadotropin. In several fish species, combined treatment of a synthetic analogue of GnRH and a dopamine inhibitor has been effective tool for overcoming reproductive dysfunction and obtaining good quality eggs in captivity[11-15, 35-37]. The combination of gonadotropin-releasing hormone analogue (GnRHa) and dopamine (DA) antagonists is not only beneficial, but also necessary for ovulation induction in teleosts. In the present study, GnRHa (Buserelin) 5 µg kg^{-1} BW in combination with DA antagonist (Metoclopramide; 5 mg kg^{-1} BW) could be effective for spawning induction in mullets. Similarly, GnRHa (Buserelin) 5 µg kg^{-1} BW in combination with DA antagonists or/and adrenergic agonist was effective for spawning induction in *Rutilus frisii kutum*[15]. Alike, other researchers have reported that GnRHa (Buserelin) alone and in higher doses was more effective in ovulation and spawning induction[38, 39].

The present results indicated that Buserelin acetate gave higher ovulation, fertilization and hatching rates than those of triptorelin acetate. The ultimate goal of hormone administration in captive reproduction of fishes is induction of FOM and ovulation, leading to either successful volitional spawning or manual stripping. Thin-lipped grey mullet in spawning experiments were given 43-48 hour window to volitionally release any ovulated eggs after which they were manually stripped. Analysis of spawning success indicated that Buserelin was effective for spawning induction in *L. ramada* at shorter latency period (43 h) than for Triptoreline. The effects of hormone induced spawning on egg quantity and quality are important considerations in determination of appropriate hormone dosage regimes. *L. ramada* females injected with Buserelin produced significantly more ovulated eggs (169±21) and fertilized eggs (54±5) per gram body weight than females injected with Triptoreline. Buserelin was effective for spawning induction in a number of fishes[15, 38-40]. Also, [D–Trp6] GnRH (Triptoreline, Decapeptyl) has been successfully used to induce final maturation and synchronize ovulation of some fish[41].

From the economic view point, the use of the pituitary gland of silver carp as a stimulant dose and also the use of Buserelin as a resolving dose is less expensive compared to the use of other tested hormones.

CONCLUSION

The number of ovulated eggs, fertilized eggs and hatched eggs were higher in buserelin acetate spawned fish than Triptoreline. Therefore this research recommends the use of the pituitary gland of silver carp and buserelin acetate in combination with Metoclopramide for effective low cost stimulation of spawning of mullets.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

تقييم فعالية وتكلفة الهرمونات المختلفة فى تحفيز تفريخ أسماك الطوبار

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المقدمة: أن لا يكتمل النضج النهائي لأسماك العائلة البورية في الأسر فلا يتم وضع البيض إلا باستخدام التحضيرات الهرمونية لتحفيز التفريخ.

الهدف من البحث: تقييم فعالية وتكلفة استخدام الأنواع المختلفة من الهرمونات؛ هرمون الغدة النخامية لأسماك المبروك, هرمون الجونادوتروبين المشيمي البشري ، الهرمونات المحررة لهرمون الجونادوتروبين في تحفيز التفريخ لأسماك الطوبار.

مواد وطرق البحث: تم إختيار الغدة النخامية لنوعين من أسماك المبروك هما المبروك العادي والمبروك الفضي وهرمون الجونادوتروبين المشيمي البشري بالاضافة إلى البوزريلين أسيتات والتريبتوريلين أسيتات لتحفيز التفريخ لأسماك الطوبار. في هذا الصدد تم اختبار تلك الهرمونات على معدل التبويض ونسبة التلقيح والتطور الجنيني ونسبة الفقس وأيضاً عمل تقييم اقتصادي لتكلفة الحقن بتلك الهرمونات.

النتائج: أظهرت النتائج فعالية الهرمونات المستخدمة في تحفيز التبويض والتلقيح والفقس ولكن بنسب مختلفة. فقد أعطى استخدام الغدة النخامية لأسماك المبروك الفضي نسبة تبويض أعلى من كل من الغدة النخامية لأسماك المبروك العادي وهرمون الجونادوتروبين البشري. أيضاً أعطى البوزريلين نسبة تبويض ونسبة تلقيح وفقس أعلى من التريبتوريلين اسيتات. أما من ناحية التقييم الاقتصادي للبرنامج أظهرت النتائج أن استخدام الغدة النخامية للمبروك الفضي كجرعة تنشيطية وأيضاً استخدام البوزريلين كجرعة متممة اقل تكلفة مقارنة باستخدام الهرمونات الأخرى التي تم اختبارها.

الإستنتاج: يمكن التوصية باستخدام الغدة النخامية لأسماك المبروك الفضي وهرمون البوزريلين لتحفيز التفريخ لأسماك العائلة البورية حيث أنهما اكثر فاعلية وأقل تكلفة.