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Spawning induction in doctor fish, *Garra rufa* (Heckel, 1843) by Ovaprim and captive rearing of larvae

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Abstract: The stimulatory effect of Ovaprim, a commercial spawning inducing agent consisting of salmon gonadotropin releasing analogue (GnRHa) and a dopamine D2- receptor antagonist, Domperidone, was studied in doctor fish, *Garra rufa* (Heckel, 1843) under hatchery conditions. The feeding and rearing condition effects on the survival rate of naturally produced larvae were also evaluated. Twenty-five fish were collected from Kohmareh stream (Helleh River), Fars province, Iran by electrofishing and transported to an ornamental fish hatchery. Fish were randomly divided into 5 groups and injected as follows: control fish received only 0.7% physiological saline (group 1) and groups 2 to 5 received 0.1, 0.2, 0.02 and 0.04 ml Ovaprim per fish. Six hours after injection, fish were evaluated for ovulation or spermiation success. None of the samples in control fish spawned until the end of experiment. Higher doses of agent in groups 2 and 3 caused a100% mortality in treated fish. In group 3, two males were spermiated but none of the females ovulated. In group 4, two ovulated fish were found whereas three fish died. Feeding larvae with newly hatched *Artemia* naupli and rearing in temperature close to 30ºC resulted in higher survival rate in comparison to fish fed a combination of phytoplankton and infusoria and compared to larvae kept in temperature lower than 24ºC or higher than 34ºC. In conclusion the results of this study showed that Ovaprim is effective in induction of spawning in doctor fish, but a dose much lower than those used in this study is recommended for use in hatchery to avoid brooders mortality. Feeding larvae with fresh *Artemia* naupli during weaning stage is also suggested for higher survival rate.

Keywords: Cyprinidae, Doctor fish, GnRHa, Induction, Ovaprim, Spawning.

Introduction

Use of exogenous hormones to induce spawning in fish species date back to 1930, when Professor B.A. Houssay reported for the first time the effectiveness of a fish pituitary extract (PE) in induction of ovulation in another fish (see Mylonas & Zohar 2001). For many years the PE, mainly pituitary obtained from ripe common carp (*Cyprinus carpio*) (CPE) was the only effective preparation for induction of ovulation and spermiation of brooders in commercial hatcheries (Vazirzadeh et al. 2011), but the expensive cost of pure and well-prepared pituitaries, limited availability worldwide and concerns regarding the potential of diseases transition from donor fish to receivers have been led to comprehensive studies on finding of new agents for induction of spawning in fish (Zohar & Mylonas 2001; Zohar et al. 2010). Primary works resulted in purifying and manufacturing of piscine gonadotropin hormones (GtH) analogues mainly carp GtH and salmon GtH which effectively used in fish hatcheries but due to high cost and antibody responses in

receiver fish, the use of pure fish GtH analogues has been limited in fish hatcheries (Zohar & Mylonas 2001). In contrast to fish GtH, human chorionic gonadotropin (hCG) now has a special role in management of fish reproduction, especially in marine and brackish water aquaculture, in fishes such as grey mullet (*Mugil cephalus*) (Vazirzadeh & Ezhdehakoshpour 2014).

In 1970, first report on the effective potential of fish synthetic gonadotropin releasing hormone (GnRHa) on GtH secretion in carp documented in France by Breton & Weil (1973). Although the application of GnRHa in salmonids hatcheries started very soon after discovering of the potency of GnRHa in induction of spawning of fish, spawning induction of carps by natural or synthetic GnRH was difficult and somewhat impossible (Breton & Weil 1973; Sokolowska et al. 1984; Peter et al. 1987). Later on, the inhibitory effect of dopamine on gonadotropin release was discovered in gold fish (Chang & Peter 1983). Thereafter, it was proved that dopaminergic inhibition can be removed by dopamine receptor antagonists (Chang & Peter 1983; Peter et al. 1987; Habibi et al. 1989). After this finding, a highly effective so called "Linpe" method was presented for spawning induction of carps using GnRH single step injection along with variety of anti-dopamines (Peter et al. 1988).

Garra rufa is a small fish belonging to the family Cyprinidae, distributing in most warm streams and shallow freshwater lakes in Iran (Esmaeili et al. 2005). This fish is known as doctor fish because of its role in cure of a dermatological disease, Psoriasis (Sayili et al. 2007). The population of doctor fish has been drastically declined in southern Iran (Esmaeili et al. 2005) as other parts of world and now is considered as an endangered or highly threatened species in many regions of the world (Sundarabarathy et al. 2005). Doctor fish is also used as ornamental fish due to its beauty and its feeding strategy which cleans the aquarium.

Conservational aquaculture is an effective method to rehabilitate the degraded aquatic ecosystems suffering from anthropogenic activities (Wildt et al. 1993; Kucharczyk et al. 2005; Sundarabarathy et al. 2005). The present experiment was conducted for the first time worldwide to study the effectiveness of a commercial GnRHa agent in induction of ovulation and spawning of an endangered species of a high recreational and ecological importance. Also, the effects of husbandry condition on larval survival of *Garra rufa* were presented.

Materials and Methods

Broodstock sampling and maintenance: The experiment on hormonal induction of spawning was carried out in an ornamental fish hatchery (El Nino) located in Marvdasht, Fars, Iran. Ripe doctor fish were collected by electroshocking from Kohmareh stream (Helleh River) Kohmareh, Shiraz, Iran in late February 2013. The physiochemical parameters of water in sampling part of the river are given in Table 1. Fish were transported to hatchery by plastic bags containing 1/3 water and 2/3 air placed in Styrofoam. Experimental aquaria (200l) were filled with infiltrated waters with 250-300 TDS. Fish were disinfected with 0.7% physiological saline before transporting to aquaria. Total weight and length of fish were measured and randomly allocated to 5 groups. To avoid stress, fish were starved for one day

Table 1. Physico-chemical parameters of the rivers where wild *Garra rufa* brooders were collected.

Date	Time	D.O. (mg/lit)	T (°C)	EC (µs/cm)	Salinity (ppt)	TDS (mg/l)	pH
17/05/2011	13:30	8.41	21.3	351	0.17	168/4	8.49
23/06/2011	11:15	7.25	25.5	663	0.32	314	8.41
20/02/2013	11:40	8.95	11.1	1153	0.58	572	7.40
22/04/2013	11:15	8.15	18.0	670	0.32	572	8.21

Table 2. The rearing condition of *Garra rufa* larvae.

Aquarium No	1	2	3	4	5	6
Feeding regime	Artemia	Artemia	Artemia	P+I*	P+I	P+I
No of Larvae	60	60	60	60	60	60
T (ºC)	30	26	24	34	32	27
pH	6.8	6.7	6.7	6.7	6.7	6.8
Water level (cm)	20	20	20	20	20	20
MDM**	5 ± 1^a	5.71 ± 1^a	5.71 ± 3^b	13.28 ± 6^d	7.14 ± 3.3^c	6.71 ± 2.5^c

*Combination of phytoplankton and Inferozoans,

** Mean daily mortality rate. Data with different letters are significantly different

before experiment. Before any handling, fish were anesthetized with 100ppm clove powder.

Hormonal treatment trials: Ovaprim is a commercially salmon GnRHa analogue which synthetized and fabricated by Syndel Co. Canada and contained 20µg [Des-Gly^{10}, D-Arg^6, Trp^7, Leu^8]-LHRH-ethylamide (sGnRHa) plus 10mg Domperidone as D2- receptor antagonist in each milliliter dissolved in glycol propylene. The recommended dose of this preparation for cyprinid fish is 0.5ml/kg body weight of fish (10µg sGnRHa and 5mg Domperidone).

For hormonal treatments, 25 fish weighed 8.56±3.21SDg were injected as follows: control fish received 0.1ml 0.7% physiological saline (group 1). Fish in groups 2 to 5 injected with 0.1, 0.2, 0.02 and 0.04ml Ovaprim per fish. Each experimental group consisted of 5 individuals. Six hour after injections, fish were checked every half an hour for ovulation and spermiation occurrence. Ovulated fish were fertilized by sperm obtained from hormonally treated males and incubated in aquaria containing artificial grasses. The temperature of water during the experiment ranged from 24-26ºC. Since the sexing of fish before hormonal therapies was not possible, both sexes were used in experiment.

Captive rearing and feeding of larvae: To study the effects of captive rearing and feeding strategy on the larval survival, an experiment was conducted in May 2011. *Garra* brooders were collected from Boshar River, Yasuj, Iran in early June 2011 when the temperature of river was 21.3ºC. Fish were immediately transported to an ornamental fish hatchery in Shiraz, Iran and treated as described before in this manuscript and placed in 200l aquaria with stable condition. Fish let to spawn spontaneously by increasing the temperature of water to 28ºC and placing disinfected small stones at the bottom of aquaria as nesting bed within 2 days after introduction. Brooders were taken after spawning and eggs were left to hatch with normal aeration to avoid hypoxia. Three days after hatching when larvae absorbing 2/3 of their yolk sac, they were divided into 6 groups each with 60 individuals kept in 20l aquaria. Fish in first three aquaria were fed 4 times a day by 0.5ml newly hatched *Artemia* naupli in each feeding time. The other 3 aquaria were fed 4 times a day with a combination of phytoplankton and infusoria. The rearing condition of each group is given in Table 2.

Statistical analyses: All data are presented as mean±S.D. except where indicated. The normality of data was tested by Kolmogorov-Smirnov. The differences between treatment groups were analyzed by one-way ANOVA, followed by Duncan using statistical software SPSS 16 at a significant level of 95%.

Results

Effects of different doses of Ovaprim in induction of spawning: The details of effects of different doses of Ovaprim in induction of spawning in *Garra rufa* are shown in Table 3. None of fish in the control group which received physiological saline spawned during the experiment, but one week after experiment some of fish in this group spawned subsequent to increasing the water temperature to 28ºC. All fish in groups receiving the higher doses of Ovaprim (0.1 and 0.2ml per fish) were died within less than 2 hours

Table 3. The effects of different doses of Ovaprim in induction of spawning of *Garra rufa*.

Treatment	Dose (ml/fish)	Mean (± S.D.) weight (g)	Spawning condition
G 1 (C)	0.1	12 ± 2.7	No spawning [a]
G 2 (O)	0.1	9.2 ± 3.24	100% mortality [a]
G 3 (O)	0.2	10.8 ± 3	100% mortality [a]
G 4 (O)	0.02	10 ± 2.19	3 males spermiating/2 females without ovulation [c]
G 5 (O)	0.04	9.2 ± 2.4	2 females ovulated/ 3 fish died [b]

C= control fish, O= fish injected with Ovaprim. Data with different letters are significantly different

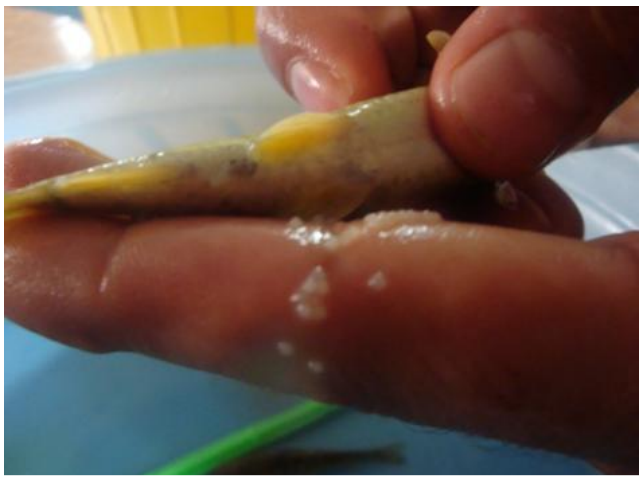

Fig.1. The effect of Ovaprim at dose 0.04ml per fish in induction of ovulation in *Garra rufa*.

after injection. The spine of fish was bended before dying showing a severe negative nervous response to the higher doses of hormone. Three male fish were spermiating in group receiving 0.02ml per fish of Ovaprim, while none of the females (2 no) in this group ovulated in response to hormonal injection. In fish injected with lowest doses of Ovaprim (0.04ml per fish) two fish ovulated (Fig. 1), whereas 3 no of fish died. Overall, the two lower doses of Ovaprim were the most effective doses in induction of ovulation and spermiation in *Garra rufa*.

Survival of larvae in different feeding regime and husbandry condition: The effects of rearing condition and feeding regime on the survival of doctor fish larvae are shown in Table 2. Fish fed by fresh *Artemia* naupli showed higher survival rate in comparison to the fish fed by combination of phytoplankton and infusoria. The results of this study also showed that temperatures higher than 34ºC and lower than 24ºC are not suitable for larval culture of this fish, because larvae in those conditions showed the highest rate of mortality. It could be concluded that a temperature range between 28-30ºC is the most likely condition for larval culturing of this species.

Discussions and Conclusions

The results showed the stimulatory effects of Ovaprim in induction of ovulation and spermiation in a native threatened cyprinid species, confirming the results of previous studies reporting the efficiency of administration of GnRHa plus dopamine antagonist in induction of reproduction in cyprinids. Treating brooders with exogenous hormonal preparations is not avoidable in almost all cyprinid commercial hatcheries (Peter & Yu 1997).

Using different synthetic GnRHa, in different ways, is a key management tool for controlling spawning in aquaculture. To date, several kinds of GnRHa have been synthetized and successfully used in different marine, brackish and fresh water species with a wide variety of gonadal developing patterns (reviewed by Mylonas et al. 2009). Amongst the tested GnRHa, mammalian (mGnRHa) and salmon (sGnRHa) are the most used analogues in aquaculture. Although in many species both of these analogues showed similar potent in induction of spawning in both sexes of fish, in some cases especially in cyprinds sGnRHa showed higher efficiency in induction of spawning and rise of GtH circulation (Zohar et al. 2010; Vazirzadeh et al. 2011).

Injection of GnRHa in liquid solutions (e.g. saline solution or glycol propylene) or incorporating of hormone in materials with slow release capacity are the most likely form of GnRHa delivery method in

fish (Mylonas & Zohar 2001; Zohar & Mylonas 2001; Mylonas et al. 2009). Some research also tested the possibility of oral administration, but with only some primary partial success which needs to be studied fruitfully (Kime et al. 1987; Solar et al. 1990; McLean et al. 1991). After discovering the potent of synthetic GnRH analogues in induction of spawning in fish, their applications in cyprinid fish, mainly in common carp and Chinese carps, were not successfully effective, until the discovery of dopamine D2-receptor antagonists ability in removal of negative effects of dopamine in GnRH stimulatory effects in pituitary (Chang & Peter 1983; Dufour et al. 2005). Subsequently, a very high effective method consisting of single injection of GnRHa combined with D2-receptor antagonists, so called Linpe method, was proposed for induced ovulation in Chinese carps (Peter et al. 1988). Later on research showed that combination of sGnRHa with Domperidone or Pimozide as D2-receptor antagonists was more effective than injection of mGnRHa and Metoclopramide (Szabó 2003; Dufour et al. 2005; Guzmán et al. 2011).

Ovaprim as a commercial stimulatory agent for control of reproduction in fish consisted of sGnRHa and Domperidone and showed to be a highly effective agent in induction of ovulation or spermiation of cyprinids with severe dopaminergic activity in hypothalamus-pituitary-gonad axis, the main axis controlling the reproduction in vertebrates (Lin et al. 1991; Yaron 1995; Kumakura et al. 2003; Glasser et al. 2004; Dufour et al. 2005). The results of our study in groups 4 and 5 that received the lower doses of Ovaprim are in agreement with previous studies regarding the efficiency of this commercial agent in reproduction control of cyprinids. Common carp treated with Ovaprim showed higher rate of ovulation in comparison to other commercial GnRHa agents (Brzuska & Adamek 1997). Injection of Indian major carps with Ovaprim resulted in higher ovulation success and fertilization rate in comparison to CPE (Rath et al. 2007). There are also some reports on the higher effectiveness of Ovaprim in induction of spawning in non-cyprinid fishes with respect to other commercial GnRHa agents. Ovaprim was more effective than Dagin (another commercial GnRHa agent in Hungary) and CPE in induction of ovulation in pike (*Esox lucius*) (Szabó 2003). Ovaprim was also highly effective in induction of ovulation and increasing circulation steroid hormones in catfish (*Heteropneustes fossilis*) in vivo and in vitro (Chaube et al. 2014). Application of Ovaprim induced the ovulation of Eurasian perch (*Perca fluviatilis*) in both cold and normal water temperatures under controlled conditions (Kucharczyk et al. 2014). In European smelt (*Osmerus eperlanus*) injection of Ovaprim led to higher spermiation rate and better sperm characteristics compared to other commercial GnRHa agent (Król et al. 2009).

Injection of doctor fish with higher doses of Ovaprim led to 100% mortality. Considering the mean weights of fish under study these doses delivered near 500 and 250µg/kg GnRHa and 250 and 125 mg/kg Domperidone, respectively, that are too much higher than the dose usually used in fish. It seems that 0.1 and 0.2ml of drug per each fish are much higher than the dose to be tolerated by fish. It already mentioned that higher doses of drugs used in control of reproduction of fish may result in severe mortality due to neuroendocrine system responses (Peter & Yu 1997). It seems that high doses of dopamine antagonist had more drastic effects than GnRHa, because higher doses of GnRHa alone showed no negative side effect in fish (Zohar et al. 2010) which needs to be evaluate. This problem was due to the calibration scale of the insulin syringe used in this study for injection of drug. Thus it is recommended to dilute the hormone in its vehicle (propylene glycol) to get the lowest effective dose when using Ovaprim in small fish.

The results also showed that feeding of *Garra rufa* larvae with newly hatched *Artemia* resulted in lower mortality rate in comparison to use combination of phytoplankton and infusoria. Studies showed that *Artemia* is the best nutritional item during the larval stage of many fish species due to its high nutritional

value, i.e., high protein, fatty acids and vitamins content (Lavens & Sorgeloos 1996). It is believed that enrichment of *Artemia* naupli may even have higher nutritional value resulting in higher survival of larvae (Ribeiro et al. 2011). The results of rearing condition also showed that larvae reared in water temperature under 24ºC and higher than 34ºC had lower survival rate. Temperature has the key role in physiology and the whole life of poikilotherm animals such as fish and controls the feeding duration and rate. Each fish has its own historical evolution pattern and has been adapted with a specific temperature range that increases foraging and exploiting the resources efficiency. Therefore, it could be concluded that since *Garra rufa* is a tropical and subtropical species and lives normally in warm waters, temperatures below 24ºC may decrease its appetite as well as digesting rate, resulting in weaker larvae which is more susceptible to environmental changes leading to higher mortality than those reared in normal temperature ranges.

In conclusion, the reproduction of *Garra rufa* could be successfully induced by a combination of sGnRHa and Domperidone. However, special care should be taken to use the lower doses of the agent to avoid mortality. Larvae fed with newly hatched fresh *Artemia* reared in temperature close to 30ºC had better survival rate. The induction of spawning protocol and rearing condition described in this research might be used in commercial ornamental fish hatchery as well as in conservational aquaculture activities to restock the natural ecosystem where *Garra rufa* is declined due to anthropogenic activities.

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