Gonadal Maturation and Spawning of Barred Loach (*Nemacheilus fasciatus*) Induced by Topical Gill Hormone Application

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Abstract

Barred loach *Nemacheilus fasciatus* are unable to reproduce naturally in captivity due to the lack of environmental cues and gonadal maturation occurs slower than in the wild. To optimize breeding procedures for this fish species, we determined whether hormone application via the gills was an effective process and assessed hormone dosage treatments. Oodev™ was used to induce gonadal maturation and Ovaprim™ was used to induce spawning via a topical gill approach. Multiple maturation parameters such as gonadal-somatic index (GSI) and hepatosomatic index (HSI), fecundity, and egg diameter; and spawning parameters such as latency period, egg produce, fertilization rate (FR), hatching rate (HR), survival rate (SR) were recorded and compared between the hormone dosage treatments and control treatment. Both the Oodev™ gonadal maturation induction and Ovaprim™ spawning induction were effectively applied to barred loach via topical gill application. Gonadal maturation parameters were positively correlated with dosage and all were significantly different. Fry survival rate was not different between doses. The optimal dose of Oodev™ (0.75 µL/g fish) resulted in male GSI of 5.334 ± 0.320%, female GSI of 15.501 ± 0.675%, male HSI of 0.416 ± 0.023%, female HSI of 1.670 ± 0.104%, egg fecundity 4584.20 ± 493.216 eggs, egg diameter 0.964 ± 0.0151 mm. Ovaprim™ optimal dosage (3.00 µL/g female and 1.50 µL/g male) was resulted latency period 11.05 ± 0.52 hours, egg produced 3504.83 ± 358.57, FR 96.77 ± 0.88%, HR 83.62 ± 3.78%, and SR 91.44 ± 2.53%.

Keywords: breeding, captivity, fish species, natural habitat, wild

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INTRODUCTION

The barred loach *Nemacheilus fasciatus* (Valenciennes, in Cuvier and Valenciennes, 1846), belonging to Cypriniformes, Nemacheilidae, Nemacheilus, is a tropical freshwater benthopelagic tiny fish found in mountain streams with rocky bottoms and clear waters on the Indonesian islands of Sumatra and Java (Froese and Pauly, 2022; Kottelat *et al*., 1993). Both habitat degradation and overfishing have diminished natural wild populations (Tjahjo *et al*., 2017; Fikri *et al*., 2023). The barred loach has the potential for economic value as a domesticated fish species in aquaculture and the aquarium trade. Small fish species provide many essential micro-nutrients as they are usually consumed whole (Kawarazuka and Béné, 2011). Developments in the domestication of barred loach are necessary to raise their number in the wild, e.g. through supplemental stocking, and meet the demands of the community in terms of fisheries and aquarium trade (Susilo *et al*., 2022).

To ensure the successful domestication of new species in aquaculture, it's crucial to manage and maintain consistent reproduction across several generations. The main objective is to enhance and ensure the production of high-quality fry without relying on wild catches. This approach aims to establish a new economic commodity, contributing to economic development and improving human livelihoods. In the wild, the reproduction of some species is driven by environmental cues, for example, flow rate changes and seasonal floods are linked as spawning triggers for many species of fish

(Baumgartner *et al*., 2014; Jenney *et al*., 2022). Therefore, some fish are unable to reproduce in aquaculture due to the loss of environmental factors that drive the development and maturation of the gonads till ovulation and spawning (Mylonas *et al*., 2010).

Previous study has shown that the maturation process of barred loach gonads in an aquarium is slower than in their natural habitat, so hormonal induction is required to accelerate and control the process (Prakoso *et al*., 2017). One of the commercial hormones commonly used to induce gonadal maturation in fish is Oodev™ (IPB University, Indonesia). Oodev™ (oocyte developer) contains pregnant mare serum gonadotropin + dopamine antagonist (PMSG+DA) that can induce an increase in the GnRH level, which in turn stimulates the pituitary to generate gonadotropin; then, gonadotropin will stimulate the ovaries to mature the eggs in the fish (Nainggolan *et al*., 2014). Oodev™ successfully facilitates gonadal maturation in catfish (*Pangasius hypophthalmus*) (Agustinus, 2013), snakehead (*Channa striata*) (Anwar *et al*., 2018), Asian redtail catfish (*Hemibagrus nemurus*) (Putri *et al*., 2019), and *Poropuntius tawarensis* (Mellisa *et al*., 2022). Meanwhile, the use of Oodev™ in inducing barred loach gonadal maturation has never been reported.

Moreover, barred loach cannot spawn naturally in culture conditions, and it has been documented to only spawn via induction by hormone injections using Ovaprim™ (Syndel Laboratories Ltd., Canada) with a dosage of 0.5–0.6 µL/g female and 0.3 µL/g male (Prakoso *et al*., 2021). Ovaprim™ is a commercial products combination of sGnRH-a (analog of the salmon gonadotropin hormone) and domperidone (dopamine antagonist) that is commonly used to induce spawning in fish (Arfah *et al*., 2006) such as silver rasbora (*Rasbora argyrotaenia*) (Ningrum *et al*., 2019), sturgeon (*Acipenser fulvescens*) (Anderson *et al*., 2013), *Clarias batrachus* (Sahoo *et al*., 2008), and many ornamental fishes (Hill *et al*., 2009).

The topical gill method is commonly used for maturation and spawning induction on tiny fish species (Hill *et al*., 2005) such as silver rasbora (*Rasbora argyrotaenia*) (Adawiyah *et al*., 2019) and rainbow shark (*Epalzeorhynchos erythurus*) (Hill *et al*., 2005). The topical gill approach has the advantage of not causing scarring and reducing stress, unlike intramuscular injection and intraperitoneal injection (Podhorec and Kouril, 2009; Fikri *et al*., 2022). The gills are an obvious conduit for the topical delivery of hormone-inducing agents (Hill *et al*., 2005). In the topical gill technique, the success of hormone absorption is influenced in part by the nature of the gill barrier between the environment and the blood through the gill lamellae, which the hormone goes through at a thickness of only 1–5 µm (Dorafshan *et al*., 2003).

According to the description of the hormone induction of fish by topical gill technique, the maturity and spawning of barred loach are expected to be induced. In addition, by the application of several doses, the optimal dose for inducing maturation and spawning in barred loach can be found. This study was conducted to identify the optimal dosage of Oodev™ induction for gonadal maturation and Ovaprim™ induction for spawning in barred loach using the topical gill technique to increase production in commercial hatcheries.

MATERIALS AND METHODS

Ethical Approval

This study was undertaken following the Law of the Republic of Indonesia No. 18 of 2002 on the National System of Research, Development, and Application of Science and Technology. This study was conducted under the ethical consideration and the approval of the Faculty of Health, Medicine, and Life Sciences, Universitas Airlangga (Letter of Assignment from Vice Dean No. 1553/UN3.1.16/KP/2022 and 1795/UN3.1.16/KP/2022.

Study Period and Location

This study was conducted from April to May 2022 at the Technical Implementation Unit for Fish and Environmental Health Laboratory of Pasuruan (UFEHLP, East Java, Indonesia).

Experimental Design

The fish used in this study were obtained from cultivation in the Fish Health and Environmental Laboratory Technical Implementation Unit, Umbulan, Pasuruan (East Java, Indonesia). This study consisted of two experiments which were carried out in parallel, the first was induction of gonadal maturation trials and the second was spawning induction experiments. Overall, we used 80 fish (40 male and 40 female) that have been spawned before for gonadal maturation treatment and 72 fish (48 male and 24 female) that have been matured for induce spawning treatment. Fish weight and size according to treatment are reported in Table 1.

The gonadal maturation of barred loach was induced using Oodev™ and spawning was induced using Ovaprim™. Applied doses of Oodev™ were the same in males and females, while the dose of Ovaprim™ given to males was half that of females. The doses of treatments are summarized in Table 2. Each Oodev™ treatment consists of 10 female and 10 male maturation, and each Ovaprim™ treatment consists of six spawning replications.

The fish were all acclimated for one week in a plastic jar with a volume of 20 liters before treatment, each jar held two individuals in sex-specific arrangements. For the maturation treatment, two individuals were kept in their sex-specific pairings for four weeks during the treatment. Oodev™ was administered by topical gill method every week following the set experimental treatment dosage (Table 2). Fish were fed sinking commercial pellets during treatment with a 3% feeding rate of fish biomass, three times a day at 09.00 AM, 12.00 AM, and 03.00 PM.

In the spawning induction treatment, both females and males were induced by the topical gill method following the set treatment dosage before spawning (Table 2). Fish broodstock (two males and one female) were then distributed into 20 liters of plastic jars with a stone substrate to replicate natural conditions at the bottom. After spawning, characterized by the presence of eggs on the substrate, broodstock was removed from spawning jars and the eggs were observed until hatched and the yolk was completely absorbed by the larvae. Water temperature, dissolved oxygen, and pH were measured during treatments are 24–28ºC, 5–8 mg/L, and 6.0–7.0.

Topical Gill Application

Hormone Oodev™ and Ovaprim™ administration followed the same topical gill procedure (Adawiyah *et al*., 2019). The hormones were diluted by 153 mM NaCl with a 1:4 ratio. The fish were anesthetized using the commercial anesthetic (Arowana Stabilizer™, Free Ocean Ltd., China) at a dose of 0.3 ppt. Then the fish were weighed individually to determine the amount of hormone used according to the treatment dose. Individual experimental doses were administered to the mouths of fish using a micropipette. The opercula was held close during application to prevent the solution from spilling out. A total of 0.7 µL/g fish NaCl 153 mM was administered to control treatment. Each fish was then placed on a plastic tray and covered with a moist paper towel for four minutes. Each fish was then placed in 20 l treatment jars following the application.

Parameters and Data Analysis

The maturation parameters observed were gonadal somatic index (GSI), hepatosomatic index (HSI), fecundity (F), egg diameter, and gonadal maturity stage (GMS). All parameters were measured and observed after four weeks or 28 days of treatments by dissecting the peritoneal cavity of all test fish. GSI, HSI, and F were obtained by the following formula:

$$\mathrm{GSI}\ (\%) = (\mathrm{GW}/\mathrm{BW}) \times 100\% \quad \ldots\ldots (1)$$

$$\mathrm{HSI}\ (\%) = (\mathrm{HW}/\mathrm{BW}) \times 100\% \quad \ldots\ldots (2)$$

$$\mathrm{F} = \mathrm{GW}/(\mathrm{GWs} \times \mathrm{TEs}) \quad \ldots\ldots (3)$$

Where GW is gonad weight, BW is body weight (g), HW is hepar/liver weight (g), GWs is gonad sample weight (g), and TEs is the total number of eggs in the sample. A total of 50 eggs were taken randomly from 10 fish in each treatment (5 eggs per fish) and were measured using a pairing Eclipse E200-LED light microscope (40×) with a video display and NIS

Elements Imaging Software (Nikon, Japan) to observe eggs diameters. The diameters of ovoid-shaped eggs were determined using the formula:

$$\sqrt{(Dxd)} \quad \text{..... (4)}$$

Where D represents the wider section of the egg and d represents the smaller section. The GMS characteristics and observation method according to Salmatin *et al*. (2021).

The spawning parameters recorded were the latency period, total number of eggs produced, fertilization rate (FR), hatching rate (HR), and larval survival rate (SR). The latency period is the time between hormonal induction and spawning. The release of eggs from the female, which were then attached to the substrate, was indicative of spawning. The latency period was determined by direct observation every hour in the mating jar. Total egg production was determined by counting the eggs on the substrate. FR (%) was determined following formula:

$$\text{FR (\%)} = (\sum \text{fertilized eggs} / \sum \text{eggs}) \times 100\% \quad \text{..... (5)}$$

Fertilized eggs seem transparent, but unfertilized eggs appear milky white. HR (%) was calculated as:

$$\text{HR (\%)} = (\sum \text{hatched eggs} / \sum \text{fertilized eggs}) \times 100\% \quad \text{..... (6)}$$

Larval SR was observed approximately 3 days after hatching (dah) until empty yolk. SR (%) larvae were obtained using the:

$$\text{SR (\%)} = (\sum \text{larvae in 3 dah} / \sum \text{hatched larvae}) \times 100\% \quad \text{..... (7)}$$

Descriptive and statistical analysis was performed on the observed parameters. The data was normally distributed after being tested with the normality test using the Kolmogorov-Smirnov test. The statistical analysis was conducted with a confidence level of 95% using one-way ANOVA then followed by post-hoc using Duncan's test and SPSS version 7.0.

RESULTS AND DISCUSSION

Based on the gonadal maturation data analysis (Table 3), we know that Oodev™ treatment by the topical gill method significantly increased all maturation parameters compared to the control (GM1) in both sexes. In the males, all gill treatments were significantly higher than the control but they were not different from each other (Table 3). The highest result for female GSI was treatment GM4 (16.448 ± 1.320%), there was a concurrent decrease relating to a reduction in dosage. HSI of males and females followed the same trend; where GM2, GM3, and GM4 were not significantly different from each other but all were significantly higher than the control (GM1). Female fecundity and egg diameter in GM1 were not observable because the oocytes were still very small in size and difficult to separate from one another, besides that the eggs were not visible. The lowest fecundity was observed in GM2 (4024.20 ± 552.335 eggs). The diameter of the eggs was not significantly different between GM2, GM3, and GM4.

Examples of dissected male and female barred loach and gonads are presented in Figure 1 and Figure 2. Based on Figure 1. the testes of the control treatment (GM1) were immature, as indicated by the white color of the sperm in the testes which were not concentrated when compared to other treatments. In Figure 2 the coloration of ovarium in GM1 treatment is darker yellow and the eggs are not visible as other treatments show that immature egg. Based on the graph and direct observation we know that the gonadal maturity stage in the control treatment was stage 2, and stage 4 in all other treatments (GM2, GM3, and GM4).

The Ovaprim™ treatment doses by the topical gill method significantly increased all parameters compared to the control treatment (Table 4.). Based on our observations, there was no spawning in the control treatment (T1), thus no parameters in T1 were observable. The fastest latency period was observed in T4 (10.45 ± 0.45 hours) and the slowest latency period occurred in T2 (11.41 ± 0.34 hours). The lowest egg

production was obtained in T2 (2576.83 ± 1144.58 egg), and there was no significant difference in egg production between T3 and T4. FR, HR, and SR were not significantly different in T2, T3, and T4.

Overall, Oodev™ and Ovaprim™ treatments proved to be successful in inducing gonadal maturation and spawning in the barred loach. We recommend its use in aquaculture and suggest optimal dosage for the species.

Inducing the Oodev™ hormone using the topical gill method in barred loach for 30 days of rearing was successful in all treatments and consistently better than the control. Oodev™ is a hormone mixture of pregnant mare serum gonadotropin (PMSG) and dopamine antagonist (Mellisa *et al*., 2022). PMSG is a glycoprotein hormone with follicle-stimulating hormone (FSH) and luteinizing hormone (LH) activity (Christakos and Bahl, 1979). FSH plays a function in early gonadal maturation or vitellogenesis, whilst LH aids in ovulation (Palermo, 2007). Dopamine antagonists can suppress dopamine, which inhibits pituitary LH secretion (Anderson *et al*., 2013). PMSG plays a critical role in fish maturity by mimicking the activities of FSH and LH, thereby stimulating gonadal development and enabling controlled breeding in aquaculture. The period of hormone activity varies, with FSH-like effects taking longer and LH-like effects being more immediate, but both are crucial for successful fish reproduction.

Individual differences in the weight of the gonads and the body mean that the GSI is highly variable. Male and female GSI increases in Oodev™ treatments than control. GSI increases with the increasing gonadal maturity stage (Mukti *et al*., 2020). GSI could be utilized to identify mature ovaries in various small, multiple-spawning fish species (Brewer *et al*., 2008). In this investigation, the GSI value was less than 20%. Therefore, it appears that barred loaches belong to the group of fish with low GSI values and can be classified as fish that can spawn multiple times per year (Mellisa *et al*., 2022). The use of Oodev™ on treatments GM2, GM3, and GM4 ripened the gonads of male brood fish, while treatment GM4 ripened the gonads of female brood fish; the gonads in these treatments are bigger than in the previous treatments, and the fish body weight also increases. The GSI is the percentage value obtained by comparing gonad weight to fish body weight (Brewer *et al*., 2008). The size and weight of the gonads generated will have reached their maximum size and weight until just before spawning occurs (Mellisa *et al*., 2022).

In the present study, Oodev™ treatment increased the Hepatosomatic Index (HSI). HSI is defined as the ratio of liver weight to fish weight and is commonly utilized in fisheries science as a biomarker of hepatic energy reserves (Cerdá *et al*., 1996). Vitellogenin (yolk precursor) synthesis, also known as vitellogenesis, occurs in the liver and is indicated by estradiol 17 β; therefore, HSI and GSI are related and their responses were expected to be similar (Hismayasari *et al*., 2015). Vitelogenin is produced in the liver, transported by the blood to oocytes, and stored as a yolk; this causes oocyte size increases, ovary weight gain, and an increase in HSI and GSI (Çek *et al*., 2001).

The fecundity of female barred loach was assessed by counting the eggs in gonads. Oosites were still very small in size in GM1 treatment and difficult to separate from one another, thus female fecundity and egg diameter were not observable. Based on all data, GM3 treatment (0.75 µL/g fish doses of Oodev™) is recommended for optimum application dose.

Ovaprim™ application in different doses with the topical gill method was successful in inducing spawning in barred loach. There was no spawning in the mating trial without Ovaprim™ application (control). In captive conditions, barred loach is only spawned by hormone injections using Ovaprim™ at a dosage of 0.5–0.6 µL/g female and 0.3 µL/g male (Prakoso *et al*., 2021). Ovaprim™ contains a combination of the hormones sGnRH-a+dopamine antagonist. The sGnRH-a hormone is a pure peptide found in teleost fish and is useful in secreting Gonadotropin Hormone II (GtH-II) or LH (Anderson *et al*., 2013). Meanwhile, dopamine antagonist inhibits the action of dopamine, and dopamine is an inhibitor of hypothalamic GnRH

Table 1. Weight and size (mean ± SD) of barred loach (*N. fasciatus*) in gonadal maturation and induce spawning treatment

Treatment	Male			Female		
	Total fish	Weight (g)	Size (cm)	Total fish	Weight (g)	Size (cm)
Gonadal maturation	40	2.18 ± 0.25	7.9 ± 0.39	40	3.00 ± 0.25	7.9 ± 0.22
Induce spawning	24	2.49 ± 0.32	7.9 ± 0.30	48	3.99 ± 0.77	7.9 ± 0.29

Table 2. Treatments doses of Oodev™ gonadal maturation induction (n = 10) and Ovaprim™ spawning induction (n = 6) of barred loach (*N. fasciatus*)

Code	Treatment	
	Doses of Oodev™ gonadal maturation induction (µL/g fish)	Doses of Ovaprim™ spawning induction (µL/g fish)
1	0.00 (control)	0.00 (control) 0.00 (control)
2	0.65	1.50 0.75
3	0.75	3.00 1.50
4	0.85	4.50 2.25

Table 3. Male and female GSI, HSI, fecundity, and egg diameter of barred loach (*N. fasciatus*)

Parameters	Gonadal Maturation (GM) Treatments dosage (µL/g fish)			
	GM1	GM2	GM3	GM4
Male GSI (%)	1.61 ± 0.09^{b}	5.33 ± 0.32^{a}	5.33 ± 0.32^{a}	5.74 ± 0.73^{a}
Female GSI (%)	7.44 ± 1.19^{c}	14.40 ± 0.56^{b}	15.50 ± 0.68^{ab}	16.45 ± 1.32^{a}
Male HSI (%)	0.25 ± 0.03^{b}	0.39 ± 0.05^{a}	0.42 ± 0.02^{a}	0.44 ± 0.08^{a}
Female HSI (%)	0.59 ± 0.11^{b}	1.64 ± 0.16^{a}	1.67 ± 0.10^{a}	1.81 ± 0.20^{a}
Fecundity	not observable	4024.20 ± 552.34^{b}	4584.20 ± 493.22^{a}	4775.40 ± 194.79^{a}
Egg diameter (mm)	not observable	0.96 ± 0.02^{a}	0.96 ± 0.02^{a}	0.97 ± 0.02^{a}

Induced maturation using Oodev™ in different doses with topical gill method and 30 rearing days (n = 10). The values are average ± standard deviation. Superscript letter in same rows presence significant differences ($p < 0.05$) between treatments. GM1 = 0.00 µL/g fish, GM2 = 0.65 µL/g fish, GM3 = 0.75 µL/g fish, and GM4 = 0.85 µL/g fish treatments.

Table 4. Latency period, egg produce, FR, HR, and SR of barred loach (*N. fasciatus*)

Parameters	Treatments (µL/g fish)			
	T1	T2	T3	T4
Latency period (hours)	not observable	11.41 ± 0.34^{b}	11.05 ± 0.52^{ab}	10.45 ± 0.45^{a}
Egg produced	not observable	2576.83 ± 1144.58^{b}	3504.83 ± 358.57^{a}	3896.50 ± 513.92^{a}
FR (%)	not observable	96.07 ± 1.84^{a}	96.77 ± 0.88^{a}	97.15 ± 1.42^{a}
HR (%)	not observable	83.32 ± 4.84^{a}	83.62 ± 3.78^{a}	83.35 ± 4.78^{a}
SR (%)	not observable	91.98 ± 1.85^{a}	91.44 ± 2.53^{a}	90.85 ± 2.34^{a}

Induced spawning using Ovaprim™ in different doses with topical gill method (n = 6). The values are average ± standard deviation. Superscript letter in same rows indicate significant differences ($p < 0.05$) between treatments. T1 = control, T2 = 1.50 µL/g female and 0.75 µL/g male, T3 = 3.00 µL/g female and 1.50 µL/g male, T4 = 4.50 µL/g female and 2.25 µL/g male.

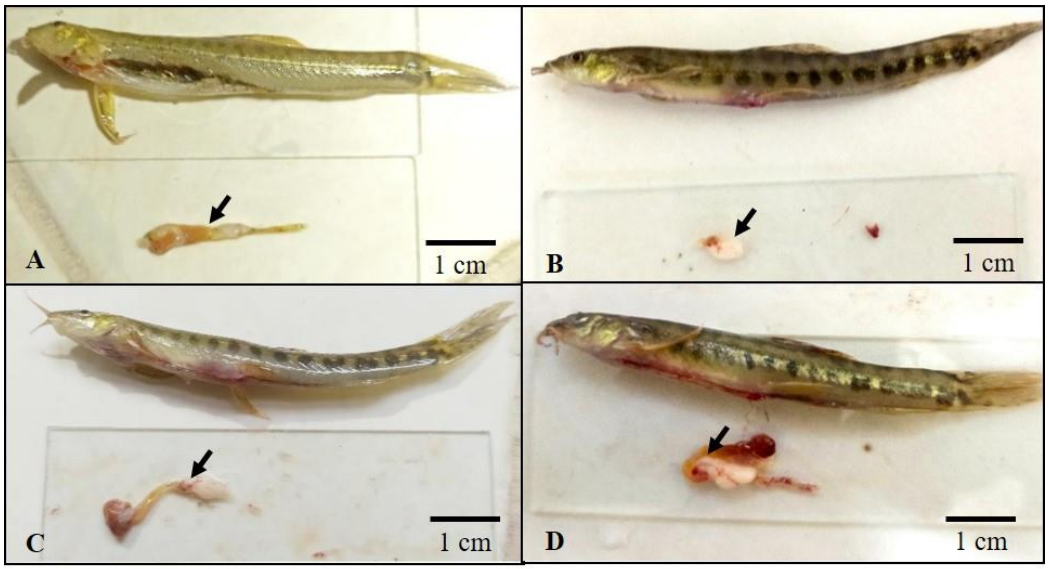

Figure 1. Testis (→) and male barred loach (*N. fasciatus*) after induced maturation using Oodev™ in different doses with topical gill method and 28 rearing days. (A) GM1 (0.00 µL/g), (B) GM2 (0.65 µL/g), (C) GM3 (0.75 µL/g), and (D) GM4 (0.85 µL/g) treatments.

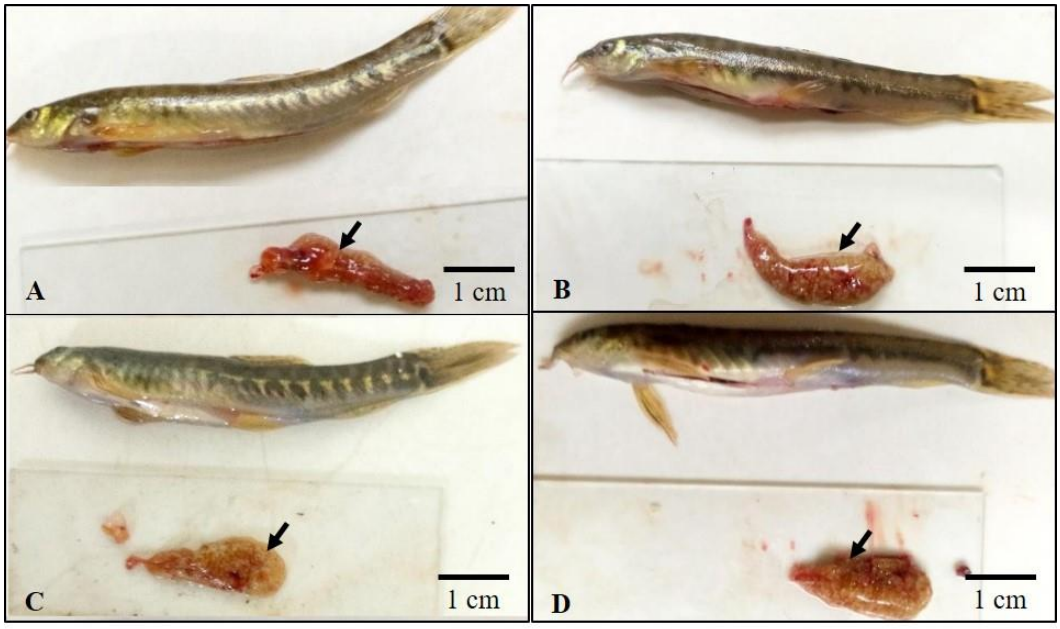

Figure 2. Ovarium (→) and female barred loach (*N. fasciatus*) after induced maturation using Oodev™ in different doses with topical gill method and 28 rearing days. (A) GM1 (0.00 µL/g), (B) GM2 (0.65 µL/g), (C) GM3 (0.75 µL/g), and (D) GM4 (0.85 µL/g) treatments.

production as a Gonadotropin Release Inhibiting Factor (GRIF) (Mañanós *et al*., 2009).

Induction with optimal doses of Ovaprim™ accelerates spawning latency time (Sahoo *et al*., 2008). Indeed, Ovaprim™ topical gill induction had a negative relation to spawning latency periods in the present study where the highest dose induced the shortest latency (4.50 µL/g female and 2.25 µL/g male dosage), showing that it is an effective technique. Spawning induction via exogenous hormone therapy at an optimal dose also shortens the time of spawning latency in striped snakehead (*Channa striata*) (Marimuthu *et al*., 2007), snow trout (*Schizothorax zarudnyi*) (Rahdari *et al*., 2014), and *Labeo rohita* (Khan *et al*., 2006).

Ovaprim™ application to the gills affected total fecundity, we suggest that T3 (3.00 µL/g female and 1.50 µL/g male dosage) is the optimal dose for efficacy as there was no difference between this and the highest test dose. The amount of LH in the body, which works to stimulate ovulation in females and spermiation in males, influences the quantity of eggs released (Cejko *et al*., 2018; Safira *et al*., 2022). In this instance, the presence of LH is altered by the interaction of sGnRH-a and dopamine antagonist included in the therapy Ovaprim™. The topical gill method requires more doses when compared to the barred loach spawning induction via injection method with a dosage of 0.5–0.6 µL/g female and 0.3 µL/g male (Prakoso *et al*., 2021; Safira *et al*., 2023). Even so, the topical gill method can be an alternative method that can be used to induce barred loach with minimum stress.

None of the experimental treatments had a detrimental effect on fertilization, hatching, or survival rates. Therefore, the use of these techniques in the aquaculture of barred loach does not negatively impact the recruitment success of fry (Muchlisin *et al*., 2007). As an applicable optimum dose, we considered using a T3 treatment dose (3.00 µL/g female and 1.50 µL/g male).

CONCLUSION

Oodev™ gonadal maturation induction and Ovaprim™ spawning induction by topical gill application have been successfully applied in barred loach. The optimum dose of Oodev™ was 0.75 µL/g fish and Ovaprim™ was 3.00 µL/g female and 1.50 µL/g male.

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AUTHORS' CONTRIBUTIONS

DSB: Conceptualization and drafted the manuscript. DSB, JS, and ATM: Validation, supervision, and formal analysis. BRA, DBR, AHF, and DSA: Performed sample evaluation. DSA and ATM: Performed the statistical analysis and the preparation of tables and figures. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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