Letters in Animal Biology

Journal homepage: www.liabjournal.com

Short-term ovarian histological changes in response to combined Ovaprim™ treatment and intra-ovarian insemination in *Barbodes binotatus*

Ikmalia Amaliy [1], Tri Ena Wulandari [2], Ragil Angga Prastiya [3]*, Darmawan Setia Budi [3], Nagia Musa Alghoul [4], Samira Musa Sasi [5]

[1] *Master's Program Student in Reproduction Biology, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia*
[2] *Faculty of Health, Medicine and Life Sciences (FIKKIA), Universitas Airlangga, Indonesia*
[3] *Department of Health and Life Science, Faculty of Health, Medicine and Life Sciences (FIKKIA), Universitas Airlangga, Indonesia*
[4] *Libyan Center of Biotechnology Research, Tripoli, Libya*
[5] *Department of Zoology, Faculty of science, University of Tripoli, Tripoli, Libya V63C+V67, Tripoli, Libya*

Article info

Received: 02 June 2026
Received in revised form: 19 August 2026
Accepted: 21 August 2026
Published online: 28 August 2026

Keywords

Barbodes binotatus
Ovaprim™
Intra-ovarian insemination
Ovarian histology
Oocyte morphometry
Phase IV oocytes

* *Corresponding author:*
Ragil Angga Prastiya
Email: ragilap@fkh.unair.ac.id

Reviewed by:

Consent to publish the names of both the reviewers could not be obtained

Abstract

Short-term ovarian responses after a combined hormonal-induction and intra-ovarian insemination procedure have not been characterized in *Barbodes binotatus*. This exploratory study evaluated ovarian histology, Phase IV oocyte diameter, and Phase IV oocyte-profile counts. Thirty-six gravid females were allocated to a natural-spawning comparator group or a combined Ovaprim™-insemination group (18 per group); three females per group were sampled at 1, 2, 3, 4, 5, and 6 h after insemination or the corresponding comparator time. Individual fish were used as the biological experimental units. Fish-level diameter data were analyzed by two-way ANOVA, whereas Phase IV counts were log(count + 1)-transformed before factorial analysis. No significant differences in oocyte diameters were found among groups ($P = 0.427$), over time ($P = 0.333$), or for the group x time interaction ($P = 0.824$). The combined-procedure group had significantly lower Phase IV profile counts ($P = 0.003$) while neither time effect ($P = 0.197$) nor the interaction ($P = 0.989$)) reached statistical significance. Collapsed follicular remnants were described as post-ovulatory follicle-like structures. Similarly, morphologically basophilic structures compatible with sperm-like material were seen. Ovulation rates, number of released eggs, fertilization, cleavage, hatching, and larval survival were not recorded in the study. Hence the results suggest an association between the combined procedure and certain ovarian-histological patterns but do not confirm treatment-specific causality, reproductive success or an optimal breeding period.

1. Introduction

Barbodes binotatus (spotted barb; locally known as wader cakul) is a small freshwater cyprinid occurring in rivers, lakes, and reservoirs across Indonesia. Inland fisheries contribute to food security and livelihoods in the countryside. However, increasing reliance on the exploitation of natural fishery resources may threaten wild populations, especially when harvesting surpasses corresponding controlled production levels (Funge-Smith and Bennett 2019; Ben-Hasan et al. 2021). Aquatic species living in freshwaters are subject to the pressures of habitat destruction, excessive exploitation, and loss of biodiversity, hence there is a strong reason to create ways of production of the species under controlled conditions rather than just relying on wild fish (Reid et al. 2019). For *B. binotatus*, field studies have shown that gonad development and reproductive activity of the species undergo seasonal variations while at the same time production on the local level is mostly still largely based on wild broodstock (Mujtahidah et al. 2019; Putri et al. 2021). Production through controlled methods necessitates breeding fish under captivity to carry out the last stages of egg cell maturation, egg release, sperm release, and gamete liberation. These biological occurrences are regulated by a system that connects the hypothalamus through the pituitary gland to the reproductive organs; specifically, gonadotropin-releasing hormone stimulates the pituitary's release of gonadotropin, which then causes gonadal steroidogenesis (Yaron et al. 2003; Zohar et al. 2010). The completion of maturation is more than just continuing the growth of the egg cell.

Ovaprim™ is a combination of a fish gonadotropin-releasing hormone (GnRH) analogue with the dopamine antagonist domperidone. The GnRH analogue stimulates gamete release by increasing the release of pituitary gonadotropins, whereas domperidone simultaneously reduces dopaminergic inhibition. GnRH-based approaches are widely used to improve reproductive control in cultured fish, although latency, ovulatory response, gamete quality, and spawning success remain species- and protocol-dependent (Sahadan et al. 2022; Roy et al. 2025). Ovaprim-associated endocrine and reproductive responses have been described in catfish and small cyprinids, but the magnitude and timing of the response vary with physiological state and administration conditions (Chaube et al. 2013; Ningrum et al. 2019). Induced spermiation has also been demonstrated in silver rasbora, whereas studies in catfish have shown that hormonal induction can facilitate ovulation under controlled conditions (Al Adawiyah et al. 2019; Sinjal 2014). Observations in another Puntius species further indicate that ovulatory characteristics cannot be

transferred uncritically among cyprinids (Suryaningsih et al. 2012).

Direct deposition of semen into the oviduct or ovarian cavity has been investigated as an alternative to conventional external fertilization. Initial studies established that sperm can be introduced into the female reproductive tract and that ovarian lavage can support induced propagation in larger fish species (Müller et al. 2018a; Müller et al. 2018b). Subsequent work demonstrated retained fertilizing capacity after ovarian insemination in African catfish and reported propagation of jundia through ovarian sperm injection (Müller et al. 2020; Ittzés et al. 2020). In zebrafish, ovarian-inseminated sperm influenced spawning success even without a male behavioral stimulus, while ovarian lavage has also been examined as a route for reproductive-hormone administration (Gazsi et al. 2021; Okomoda et al. 2023). Thus, the novelty of the present study is not the development of ovarian insemination per se, but the short-term histo-morphometric characterization of *B. binotatus* after the combined procedure.

Histology can serve as short-term documentation of oocyte development, follicular morphology, and follicular remnants following follicular rupture. In standardized teleost literature terminology, the oocyte stages that are not affected by ovulation are referred to as intact oocytes; the terms post-ovulatory follicle are reserved for follicles where ovulation occurred. However, atretic follicles can be collapsed or irregular and may morphologically overlap with post-ovulatory remnants in routine sections, making definitive differentiation difficult (Brown-Peterson et al. 2011; Corriero et al. 2021). Furthermore, teleost ovaries are generally capable of containing several oocyte cohorts, as well as considerable within- and among-fish heterogeneity, and hence a single histological section essentially provides only a limited view of a dynamic reproductive process (Serrat et al. 2019; Adolfi et al. 2023). Histological observations therefore require cautious interpretation and should not be treated as substitutes for direct measurements of egg release, fertilization, or hatching.

For *B. binotatus*, the short-term ovarian pattern following a combined Ovaprim™ and intra-ovarian insemination procedure has not been characterized using fish-level histomorphometric data. The present exploratory study evaluated Phase IV oocyte diameter, Phase IV oocyte-profile counts, follicular remnants, and the distribution of basophilic sperm-like structures during the first 6 h after insemination. It was expected that the combined procedure would be associated with fewer retained Phase IV profiles, but not necessarily with a measurable increase in Phase IV diameter. Because the design did not separate hormonal induction from insemination and did not measure direct reproductive outcomes, the study was intended to identify histological associations and priorities for a definitive follow-up experiment rather than to establish treatment efficacy or an optimal breeding time.

2. Materials and Methods

2.1. Ethical approval

All procedures involving live fish were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga (Certificate No. 2.KEH.143.10.2024).

2.2. Experimental design and biological replication

The experiment used a completely randomized, destructive sampling design and was carried out from September to November 2024. a total of 36 gravid females were divided into two groups – a natural-spawning control group (n = 18) and a combined-procedure group (n = 18) that was administered Ovaprim™ followed by intra-ovarian insemination (n = 18). Three females from each group were sacrificed at each of six observation times (1- 6 h). Therefore, each treatment x time cell contained three biological replicates and a total of six fish were sampled at each time. The individual fish was the experimental unit; oocytes and microscopic fields within a fish were nested subsamples and were not treated as independent replicates. Females in the comparator group were maintained with mature males at a ratio of 1:2. In the combined-procedure group, mature males served as semen donors. A total of 54 males were used – 36 as spawning partners in the comparator group and 18 as semen donors. The limited replication (n = 3 per treatment x time cell) was not based on a prospective power calculation and has been acknowledged in the interpretation. Since the experimental group was subjected to hormonal induction and intra-ovarian insemination, the present design aimed to evaluate the combined procedure rather than discriminating between the effects of hormone treatment, mechanical insemination or semen deposition.

2.3. Broodstock selection, acclimatization, and feeding

Broodfish were obtained from freshwater sources in Banyuwangi and Probolinggo, Indonesia. Females were selected based on abdominal distension and the release of yellowish oocytes after gentle abdominal examination. Males were considered mature when gentle abdominal stripping produced milky-white semen. Female body weight was approximately 12 g, and total length was 7-8 cm. The archived records describe broodstock generally within this size range but do not contain sex-specific male mean ± SD; values from another species, population, or experiment were not substituted. Fish were acclimatized for seven days in aerated 30 × 20 × 20 cm aquaria. They were fed twice daily, morning and afternoon, according to a standardized feeding protocol with commercially available frozen Chironomus sp. larvae and commercial fish pellets. Aquaria were cleaned daily or according to condition. Water was aerated and maintained under the ambient laboratory photoperiod; continuous water-quality logging was not performed.

2.4. Ovaprim™ preparation and administration

Ovaprim™ injectable solution (Western Chemical Inc., Ferndale, WA, USA; 20 μg mL^{-1} salmon GnRH analogue and 10 mg mL^{-1} domperidone) was administered intramuscularly at 0.7 mL kg^{-1} body weight to females for ovulatory induction and to donor males for spermiation induction (Fig. 1). The commercial solution was diluted tenfold immediately before administration by mixing one volume of Ovaprim™ with nine volumes of aquabidest. Consequently, the commercial-product dose for a 12 g fish was 8.4 μL, which corresponded to approximately 84 μL of working solution. The working solution was drawn into a 1 mL OneMed syringe with 0.01 mL graduations and divided between the right and left posterior dorsal musculature. The dilution increased the working volume and reduced the relative measurement error associated with directly administering an 8.4 μL undiluted dose. The product lot number was not legible in the archived vial image.

2.5. Semen collection and intra-ovarian insemination

Semen was collected from mature donor males by gentle abdominal stripping approximately 9.5 h after hormonal induction. Samples were pooled, protected from contact with water, and used without an extender. Intra-ovarian insemination was performed 10 h after hormone administration. The semen dose was 0.5 mL kg^{-1} body weight (0.5 μL

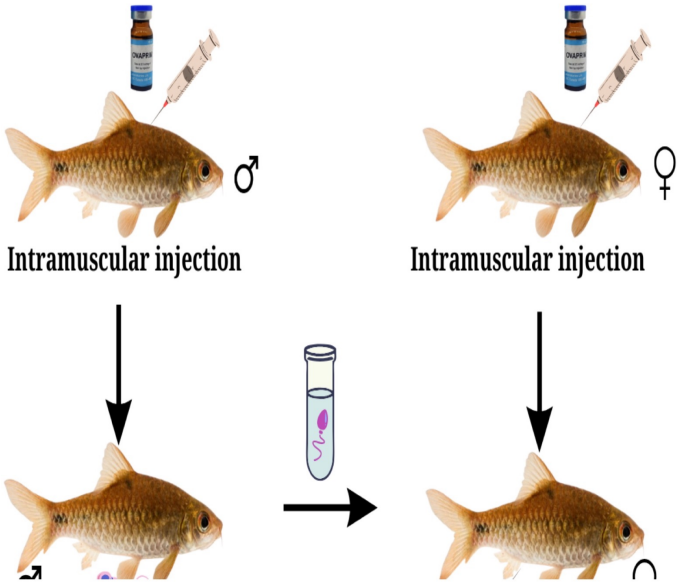

Fig. 1. Experimental workflow. Mature males and females received Ovaprim™; semen was collected from donor males and 6 µL was deposited through the genital papilla of an approximately 12-g female using a calibrated 2-20 µL micropipette tip. The diagram is schematic and not to scale

g^{-1}). Therefore, a 12 g female received 6 µL of semen. Semen was deposited into the ovarian cavity using a calibrated Finnpipette™ F1 micropipette fitted with a Thermo Scientific Finntip™ 20 tip introduced approximately 4 mm through the genital papilla of an anesthetized female. No evaluation of semen quality or fertilizing capacity was performed because, sperm concentration, motility, and viability were not measured.

2.6. Sampling, tissue processing, and histology

Throughout each observation period, fish were heavily sedated with Koi Anesthesia (KEKO™) before being subjected to necropsy. Ovaries were surgically removed via a ventral abdominal slit. The contemporary records document 24 h immersion in fixative followed by routine paraffin embedding. In preparation for microscopic histology (especially of delicate ovarian material), the tissue underwent sectioning at 4 µm followed by mounting, hematoxylin and eosin staining, and fixation in 10% neutral buffered formalin. However, it is important to mention that ovaries were collected destructively and that observations at different time points were obtained from different fish. Therefore, temporal comparisons were cross-sectional rather than longitudinal within individuals.

2.7. Histological staging, morphometry, and image verification

Oocyte profiles were classified as primary-growth, cortical-alveolar, vitellogenic, or late-vitellogenic/Phase IV profiles using morphological criteria adapted from standardized teleost terminology (Brown-Peterson et al. 2011). Collapsed follicular remnants lacking intact ooplasm were recorded separately as post-ovulatory follicle-like (POF-like) structures and were not treated as a fifth developmental phase of an intact oocyte. This terminology was adopted because atretic follicles, post-ovulatory remnants, and processing artefacts can overlap morphologically in routine H&E sections. Phase IV oocyte diameter was calculated as the geometric mean of the longest and shortest axes {√(D × d)}. Archived morphometric images retained calibrated measurement overlays in micrometres. The contemporaneous records document an Olympus trinocular microscope at 40× total magnification for morphometry and a Nikon Eclipse E200 trinocular microscope at 40×, 100×, and 1000× total magnification for complementary histological assessment. The figures were prepared only from archived panels that retained calibrated overlays, an original scale bar, or documented magnification. Representative non-overlapping fields were examined and the fish-level summaries recovered from the raw dataset were used for statistical analysis.

2.8. Statistical analysis

The archived fish-level dataset was reanalyzed. For Phase IV diameter, a two-way fixed-effects ANOVA was fitted with group, observation time, and the group x time interaction. Residual normality and homogeneity were evaluated with Shapiro-Wilk and Levene tests. Raw Phase IV profile counts showed non-normal residuals; counts were therefore transformed as log (count + 1) before the same factorial ANOVA. Effect magnitude was reported as partial eta squared (ηp^2), with exact P values. Per-time group means, standard deviations, mean differences, and 95% confidence intervals are presented descriptively; separate repeated significance tests at each time were not used for the primary inference. Analyses were conducted at the fish level with $\alpha = 0.05$.

3. Results

3.1. Phase IV oocyte diameter

Across all observation times, the mean Phase IV diameter was 424.11 ± 65.03 µm for the natural-spawning group compared with 403.73 ± 81.49 µm for the combined procedures group (Table 1). The raw distributions overlapped extensively with the comparator fish values spanning 210.87 to 459.76 µm while combined-procedure fish had values in a range of 210.02 to 526.39 µm. Factorial analysis confirmed there was no overall group effect ($F_{1,24} = 0.652$, $P = 0.427$, $\eta p^2 = 0.026$), time effect

Table 1. Phase IV oocyte diameter (µm) at the fish level (n = 3 per group per time)

Time	Comparator	Combined procedure	Mean difference	95% CI
1 h	429.48 ± 36.44	373.50 ± 39.78	-55.98	-142.45 to 30.49
2 h	422.84 ± 43.31	355.26 ± 127.60	-67.58	-283.59 to 148.42
3 h	380.21 ± 149.39	377.34 ± 81.02	-2.87	-275.29 to 269.55
4 h	427.73 ± 23.13	393.15 ± 73.37	-34.57	-157.89 to 88.75
5 h	409.58 ± 46.50	451.77 ± 70.09	42.19	-92.64 to 177.02
6 h	474.83 ± 24.41	471.36 ± 72.78	-3.47	-126.52 to 119.58

($F_{5,24}$ = 1.214, P = 0.333, ηp^2 = 0.202), or interaction of group and time ($F_{5,24}$ = 0.429, P = 0.824, ηp^2 = 0.082). The group difference did not show the same direction across time. Mean diameter was less in the combined-procedure group at 1 to 4 h but greater at 5 h and almost the same for both groups at 6 h. The 95% confidence intervals for the time-specific between-group differences included zero, indicating no evidence of an interaction,. Furthermore, the estimates had limited precision because only three biological replicates per treatment x time cell were available. No significant deviations from homogeneity of variance or normality were indicated by the Shapiro-Wilk and Levene tests.

3.2. Phase IV oocyte-profile count

Raw Phase IV profile abundance averaged 71.89 ± 53.07 profiles per fish in the comparator group and 25.33 ± 20.56 profiles per fish in the combined-procedure group (Table 2). Thus, the untransformed descriptive mean in the combined-procedure group was 46.56 profiles lower and represented approximately 35.2% of the comparator mean. Following log(count +1) transformation, a between-group effect was observed ($F_{1,24}$ = 10.900, P = 0.003, ηp^2 = 0.312) based on factorial analysis of the data, whereas neither a significant change in the outcome parameter over time (the time effect: $F_{5,24}$ = 1.604, P = 0.197, ηp^2 = 0.250) nor group-by-time interaction was significant (group-by-time interaction effect: $F_{5,24}$ = 0.110, P = 0.989, ηp^2 = 0.022). The distribution of the residuals after applying this transformation met the assumption of normality, as Shapiro-Wilk test showed that the assumption was fulfilled (Shapiro-Wilk test P = 0.246).

Although the mean level in the combined-procedure and comparator groups remained relatively stable across observation times with the lowest mean occurring in the combined-procedure group at all observation times, the magnitude of the raw difference was highly variable among them. The largest descriptive between-group difference occurred at 4 h, while comparator counts at this time were highly fluctuating. Also, the 6-h interval-specific confidence interval was the only one that excluded zero. The presence of such a pattern was, however, not sufficient to interpret either the 4-h or 6-h as response-peak times because the pre-specified factorial analysis did not confirm that the group difference fluctuated over time. As a result, we can regard the primary finding of our study as the identification of an overall between-group association rather than detecting a discrete post-insemination threshold.

3.3. Descriptive histological findings

Ovarian sections from both groups contained multiple developmental stages, including primary-growth, cortical-alveolar, vitellogenic, and late-vitellogenic profiles (Fig. 2; Fig. 3). Archived combined-procedure panels additionally contained collapsed or folded follicular remnants

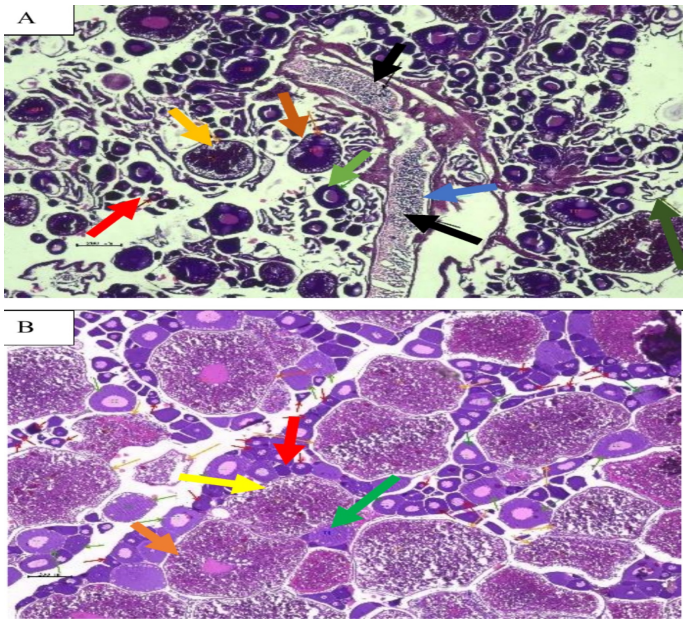

Fig. 2. H&E ovarian panels from the combined-procedure group (A) and natural-spawning comparator group (B). Colored arrows identify representative oocyte stages, ovarian lumen, follicular remnants, and basophilic sperm-like structures according to the original histology record. Both panels were documented at 40× total magnification; panel A retains the original 200-µm scale bar

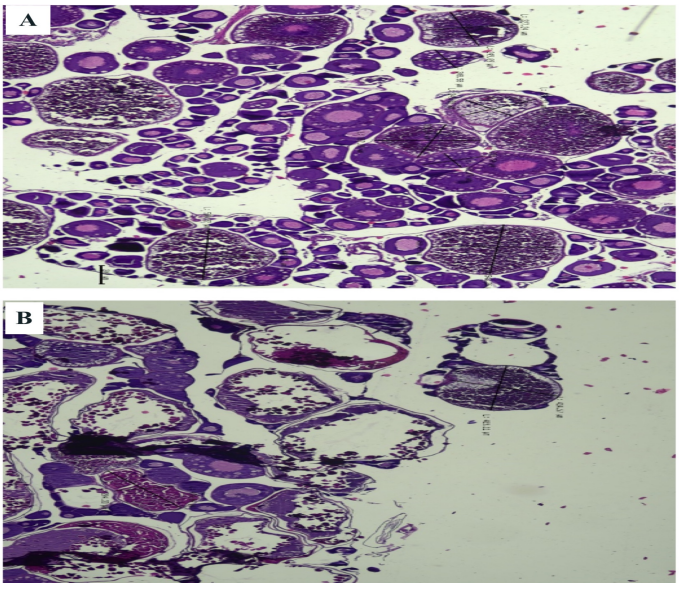

Fig. 3. Calibrated Phase IV oocyte morphometry panels. (A) Comparator section and (B) combined-procedure section at 6 h. Original micrometre measurement overlays are retained; panel A also retains the original 200-µm scale bar

Table 2. Phase IV oocyte-profile counts at the fish level (n = 3 per group per time)

Time	Comparator	Combined procedure	Mean difference	95% CI
1 h	59.33 ± 26.63	26.66 ± 16.55	-32.66	-82.95 to 17.60
2 h	62.67 ± 33.51	37.33 ± 44.27	-25.33	-114.33 to 63.66
3 h	70.66 ± 56.48	16.66 ± 8.02	-52.00	-145.05 to 41.05
4 h	115.68 ± 103.27	31.66 ± 7.22	-84.00	-249.94 to 81.94
5 h	26.33 ± 23.98	9.66 ± 8.08	-16.67	-57.21 to 23.89
6 h	96.68 ± 16.93	28.00 ± 21.28	-68.66	-112.25 to -25.09

with markedly reduced or absent intact ooplasm. These profiles were classified conservatively as POF-like structures because routine H&E morphology did not allow unequivocal separation of post-ovulatory remnants from advanced atresia or processing artefacts. Basophilic ovoid structures morphologically compatible with sperm-like material were observed within the ovarian lumen and among ovarian lobules in the combined-procedure material. Dense heads were more readily visible than flagella, and no sperm-specific stain, motility assessment, or viability assay was performed. Their distribution was therefore interpreted as qualitative evidence consistent with deposition and short-term retention of sperm-like material, not as proof of functional sperm transport or fertilization. Ovulation was not verified by strip, counting of released eggs, or direct observation of egg release. *B. binotatus* is an externally fertilizing fish so the presence or absence of cleavage-stage embryos in ovarian sections was not regarded as a stage. The fertilized and non-fertilized eggs, cleavage, embryo-development, hatchability, and survival of the juvenile were not determined; therefore, the reproductive success could not be assessed by using histology alone.

4. Discussion

Phase IV oocyte diameter was not associated with group, observation time, or their interaction, whereas Phase IV profile abundance was lower overall in the combined-procedure group. The absence of a group x time interaction is central: the data do not support a distinct acceleration point, peak ovulatory response, or optimal breeding time within the 1-6 h observation window. The result is therefore an overall histological association between groups rather than evidence that a specific post-insemination hour was biologically superior. The group effect for transformed Phase IV counts had a partial eta-squared of 0.312, indicating a substantial statistical association within this dataset. Nevertheless, effect magnitude must be interpreted together with the small biological sample, broad confidence intervals, and marked dispersion of raw counts. Comparator fish ranged from 6 to 227 Phase IV profiles, showing that ovarian status differed considerably among individuals before any time-specific inference could be made. Variation in reproductive condition is common in fish populations and can remain pronounced even when animals are sampled during a common reproductive period (Dobre et al. 2026).

The absence of a detectable change in oocyte diameter has several possible biological explanations. The diameter of a late-vitellogenic oocyte primarily reflects accumulation of yolk and the growing cytoplasm, whereas maturation involves GV migration and other changes, such as steroid signaling, water uptake, and follicle breakdown, that may occur without significant expansion of the histological profile (Mahalingam and Santhanam 2023; Tang et al. 2020). Changes associated with maturation are regulated by gonadotropin signaling through the reproductive endocrine axis, rather than by continued vitellogenic growth (Yaron et al. 2003; Zohar et al. 2010). The combined-procedure group had a modestly lower overall raw mean diameter, but the group effect was small and statistically unsupported. Time-specific mean differences also changed direction, and the 6 h means were almost identical. This pattern argues against a progressive treatment-related enlargement or shrinkage during the short observation period. It is compatible with reports that induced reproductive events can occur without a parallel increase in mature-oocyte size, although the relevant latency and response depend on species and induction protocol (Ningrum et al. 2019; Eslamizadeh et al. 2024). The present analysis cannot determine whether nuclear maturation or maturation-inducing steroid production occurred because germinal-vesicle breakdown, plasma hormones, and ovarian steroid concentrations were not measured. Endocrine explanations should therefore be treated as biological context rather than demonstrated mechanisms. The broad confidence intervals further indicate that moderate changes could have remained undetected with only three fish in each treatment-time cell.

Fewer retained Phase IV profiles may be compatible with progression beyond the late-vitellogenic stage, particularly when follicular remnants are present, but other possible explanations cannot be ruled out. Standardized histological frameworks use post-ovulatory follicles as retrospective evidence that follicular rupture has occurred, while oocyte-frequency distributions can provide information on reproductive development (Brown-Peterson et al. 2011; Serrat et al. 2019). However, the present count outcome is not an ovulation rate because neither the number of released eggs nor the proportion of females that ovulated was recorded.

Other possible explanations should be taken into account as well. The ovaries of teleost fishes consist of asynchronous oocyte groups and fish that appear to have the same maturity stage externally may actually differ in their ovarian developmental composition and endocrine responsiveness (Adolfi et al. 2023). The presence of advanced atresia can decrease the number of undamaged late-vitellogenic profiles and can also create folded follicular structures that in routine H&E sections look like post-ovulatory remnants (Corriero et al. 2021). The differences in section plane, tissue area, or number of fields counted could also influence the profile counts because there was no fully standardized sampling denominator preserved with the archived protocol. The temporal pattern reinforces the need for caution. Raw means were lower in the combined-procedure group at all six times, but the group difference did not vary statistically over time. The large 4 h difference was driven partly by a high and variable comparator mean, whereas the narrower 6 h contrast cannot be interpreted as an isolated effect after examining six time points. The factorial model therefore provides stronger support for a general group separation than for the original claim of accelerated ovulation at 6 h.

Ovaprim™ is supposed to act via its GnRH analogue by inducing pituitary gonadotropin release, whereas domperidone acts by reducing dopaminergic inhibitory effects. Previous literature and experimental studies have shown that GnRH-based stimulation has been used as a means of controlling egg maturation and ovulation, but the results are influenced by broodstock quality, dosage, environmental factors, and target species (Sahadan et al. 2022; Roy et al. 2025). Administration of endocrine substances can cause disruption at the level of sex steroid synthesis, follicle development, and ovulation activity. However, such consequences are to be measured directly with endocrine or reproductive methods (Eslamizadeh et al. 2024). Evidence from catfish and cyprinids confirms that Ovaprim can affect reproductive physiology, but it does not justify attributing the present histological difference solely to the hormone (Chaube et al. 2013; Ningrum et al. 2019). The protocol simultaneously included hormonal induction of females, spermiation induction of donor males, handling, insertion of a micropipette tip, and deposition of undiluted semen. Any of these components, or their interaction, could have contributed to ovarian appearance and the number of retained Phase IV profiles. Induced spermiation in small cyprinids also depends on administration route

and male responsiveness, neither of which was evaluated quantitatively here (Al Adawiyah et al. 2019). A definitive causal design would therefore require at least four groups: an untreated or handling comparator, Ovaprim-only, sham-insemination or insemination-only, and the combined procedure. Randomization should be documented, baseline maturity should be standardized, and the sample size should be prospectively calculated for the treatment-by-time interaction. This design would permit separation of hormonal, mechanical, semen-related, and combined effects.

Collapsed follicular profiles were consciously labeled as POF-like because routine H&E staining technique differentiate unequivocally between post-ovulatory remnants, atretic follicles, and processing artefacts (Brown-Peterson et al. 2011; Corriero et al. 2021). If post-ovulatory follicles are going to be reliably identified, the sectioning plane and sampling interval should be fixed along with the use of calibrated scale bars and the diagnosis should be confirmed by multiple blinded observers. In addition, quantitative reporting distinguishing POF-like structures from atretic follicles would provide better interpretation. Furthermore, follicular profiles expressed as the count of follicles per unit tissue area would also be the most informative report. Although the small, basophilic, rounded cells in the cross-sections closely resembled sperm, they could not be identified unambiguously by light microscopy of H&E sections. No additional sperm-specific markings methods, such as fluorescent or immune-histochemical staining, computer-assisted sperm motility analysis, concentration measurement or cell viability testing, were carried out during this study. In the present context, the visible small male gametes would be consistent with the procedural findings which indicate that only sperm-like material was introduced. In such a scenario, it is difficult to argue that the spermatozoa remained motile, reach the ovulated eggs, and fertilize them. This evidential boundary differentiates the present work from previous ovarian-insemination studies. Direct injection into the oviduct or ovary and ovarian lavage were developed with subsequent assessment of induced propagation or reproductive outcomes (Müller et al. 2018a; Müller et al. 2018b). Later studies tested fertilizing capacity in African catfish and obtained offspring after ovarian sperm injection in jundia (Müller et al. 2020; Ittzés et al. 2020). Zebrafish experiments assessed spawning success, while ovarian lavage research evaluated a reproductive intervention using direct performance endpoints (Gazsi et al. 2021; Okomoda et al. 2023). The present contribution is therefore narrower: it documents short-term, species-specific ovarian histology after the combined procedure without claiming successful propagation.

At the hatchery, the clinically important reproductive outcomes in this context are the ovulation rate of females, interval between administration of hormones and spawning, the number and quality of retrieved eggs, the rate of fertilization and the number of cells formed from the developing eggs, hatchability, and larval survival. Timing is important because prolonged storage of mature or ovulated oocytes can reduce egg quality but the optimal interval varies from species to species (Samarin et al. 2017). Quality of feed and the condition of broodstock level can influence gamete quality and performance; therefore, these aspects should be included in protocols (Bera et al. 2025). A subsequent experiment should measure semen volumes, sperm concentration, motility, viability, and the number of sperm delivered per female. Only after evaluating reproductive outcomes directly under a standardized hatchery protocol can practical breeding advice be issued (Ode et al. 2024). The study nevertheless has identifiable strengths. The fish-level dataset was recovered and reanalyzed using a factorial model that reflects the treatment and time structure, the semen-dose unit was corrected from contemporaneous records, and calibrated archived panels were used rather than retrospectively estimated scale bars. Reporting the fish as the biological replicate also avoids treating oocytes or microscopic fields as independent animals. These corrections improve the traceability of the quantitative findings, while the explicit limitations prevent the preliminary evidence from being overstated. The remaining limitations are substantial: three fish per treatment-time cell, no prospective power calculation, no baseline histological sampling, combined treatment components, unmeasured semen quality, incomplete male morphometrics, incomplete field-level sampling records, and absence of direct reproductive outcomes. These constraints limit causal, temporal, and practical inference. They do not negate the observed group association, but they define the study as an exploratory basis for a larger, prospectively designed experiment.

5. Conclusions

The combined Ovaprim™ and intra-ovarian insemination procedure was associated with lower Phase IV oocyte-profile counts than the natural-spawning comparator, whereas Phase IV diameter did not differ by group or time. The absence of a group-by-time interaction precludes identification of a specific optimal observation or breeding time. The POF-like and sperm-like structures were descriptive findings only. Because ovulation, fertilization, hatching, and offspring production were not measured and the treatment components were not separated, the study does not establish reproductive success or a treatment-specific mechanism. The findings provide preliminary, verifiable histological data for designing a larger factorial study in *B. binotatus*.

Declarations

Funding: This study received no external funding

Conflict of interest: The author declare no conflicts of interest

Acknowledgements: None

Ethics approval: Described in the Materials & Methods section

Data availability: The fish-level dataset used for the analysis is given in Supplementary Table S1. Additional archived histological panels and statistical outputs are available from the corresponding author on reasonable request

Declaration AI-assisted editing: Generative AI was used for language editing and organization of the manuscript. It was not used to generate experimental data, alter observations, or conduct unverified statistical analyses. All source-data corrections, methodological clarifications, and interpretations were checked and approved by the authors, who remain accountable for the manuscript

References

Adolfi MC, Depincé A, Wen M, Pan Q, Herpin A. (2023). Development of ovaries and sex change in fish: Bringing potential into action. Sexual Development 17(2-3): 84-98. https://doi.org/10.1159/000526008

Al Adawiyah L, Sulmartiwi L, Bodur T, Budi DS. (2019). Induction of spermiation using Ovaprim™ with topical gill method in the silver rasbora *Rasbora argyrotaenia*. Theriogenology 126: 172-176.

https://doi.org/10.1016/j.theriogenology.2018.12.014

Ben-Hasan A, Walters C, Hordyk A, Christensen V, Al-Husaini M. (2021). Alleviating growth and recruitment overfishing through simple management changes: Insights from an overexploited long-lived fish. Marine and Coastal Fisheries 13(2): 87-98. https://doi.org/10.1002/mcf2.10140

Bera P, Dhara H, Mandal S, Sau SK, Das S. (2025). Nutritional strategies for improving reproductive performance in fishes. In: Ahmed I, Ahmad I, editors, Aquaculture: Enhancing Food Security and Nutrition. Springer Nature Switzerland, Cham. Pp. 409-430. https://doi.org/10.1007/978-3-031-92858-1_20

Brown-Peterson NJ, Wyanski DM, Saborido-Rey F, Macewicz BJ, Lowerre-Barbieri SK. (2011). A standardized terminology for describing reproductive development in fishes. Marine and Coastal Fisheries 3(1): 52-70. https://doi.org/10.1080/19425120.2011.555724

Chaube R, Singh RK, Joy KP. (2013). Effects of Ovaprim, a commercial spawning inducer, on vasotocin and steroid hormone profiles in the catfish *Heteropneustes fossilis*: In vivo and in vitro studies. General and Comparative Endocrinology 195: 190-200. https://doi.org/10.1016/j.ygcen.2013.11.009

Corriero A, Zupa R, Mylonas CC, Passantino L. (2021). Atresia of ovarian follicles in fishes, and implications and uses in aquaculture and fisheries. Journal of Fish Diseases 44(9): 1271-1291. https://doi.org/10.1111/jfd.13469

Dobre A, Stroe MD, Lambert P, Dima FM. (2026). Preliminary data regarding the physiological state of *Alosa immaculata* (Bennett, 1835) during spawning migration. Aquaculture, Fish and Fisheries 6(1): e70188. https://doi.org/10.1002/aff2.70188

Eslamizadeh E, Mabudi H, Roomiani L, Baboli MJ, Dezfulnejad MC. (2024). Effects of thyroxin and luteinizing hormone releasing hormone on reproductive physiology of rohu *Labeo rohita*: insights into spawning performance, oocyte maturation, steroidogenesis, and follicular development genes. Animal Reproduction Science 267: 107542. https://doi.org/10.2139/ssrn.4761709

Funge-Smith S, Bennett A. (2019). A fresh look at inland fisheries and their role in food security and livelihoods. Fish and Fisheries 20(6): 1176-1195. https://doi.org/10.1111/faf.12403

Gazsi G, Butts IAE, Zadmajid V, Ivánovics B, Ruffilli L, Urbányi B, Csenki Z, Müller T. (2021). Ovarian inseminated sperm impacts spawning success in zebrafish, *Danio rerio* (Hamilton, 1822) even in the absence of a male stimulus. Theriogenology 172: 315-321. https://doi.org/10.1016/j.theriogenology.2021.07.012

Ittzés I, Kronbauer EC, Szabó T, Horváth L, Urbányi B, Müller T. (2020). Propagation of jundia *Rhamdia quelen* (Siluriformes: Heptapteridae) by applying the ovarian sperm injection method. Aquaculture Reports 16: 100275. https://doi.org/10.1016/j.aqrep.2020.100275

Mahalingam A, Santhanam P. (2023). Vitellogenesis and reproductive strategies in fishes. In: Baskaralingam V, Vanichviriyakit R, editors, Vitellogenin in Fishes – Diversification, Biological Properties, and Future Perspectives. Springer Nature Singapore, Singapore. Pp. 105-121. https://doi.org/10.1007/978-981-99-5340-0_8

Mujtahidah T, Marsoedi M, Widodo MS. (2019). The reproductive cycle of *Puntius binotatus* on the middle of the rainy season. Indonesian Journal of Tropical Aquatic 2(1): 9-15. https://doi.org/10.22219/ijota.v2i1.9678

Müller T, Horváth L, Szabó T, Ittzés I, Bognár A, Faidt P, Urbányi B, Kucska B. (2018a). Novel method for induced propagation of fish: Sperm injection in oviducts and ovary/Ovarian lavage with sperm. Aquaculture 482: 124-129. https://doi.org/10.1016/j.aquaculture.2017.09.025

Müller T, Kucska B, Horváth L, Ittzés Á, Urbányi B, Blake C, Guti C, Csorbai B, Kovács B, Szabó T. (2018b). Successful, induced propagation of African catfish (*Clarias gariepinus*) by ovarian lavage with sperm and hormone mixture. Aquaculture 485: 197-200. https://doi.org/10.1016/j.aquaculture.2017.11.051

Müller T, Ács É, Beliczky G, Makk J, Földi A, Kucska B, Horváth L, Ittzés Á, Hegyi Á, Szabó T, Urbányi B, Quyen NN, Orbán L, Havasi M. (2020). New observations about the fertilisation capacity and latency time of sperm inseminated into the ovary of African catfish (*Clarias gariepinus*), an oviparous modelfish. Aquaculture 522: 735109. https://doi.org/10.1016/j.aquaculture.2020.735109

Ningrum DRK, Budi DS, Sulmartiwi L. (2019). Induksi ovulasi ikan (*Rasbora argyrotaenia*) menggunakan Ovaprim™ dengan dosis berbeda. Depik 8(2): 117-124. https://doi.org/10.13170/depik.8.2.14076

Ode I, Rahmadiah T, Abdullah N, Krettiawan H, Alimaturahim F, Rumondang A, Budi DS, Puspaningsih D, Juharni, Nizar M. (2024). Teknologi reproduksi dan pembenihan ikan. Kamiya Jaya Aquatic Press.

Okomoda VT, Amighty RO, Bem TM, Amaantimin J, Nurizzati I, Koh ICC, Abol-Munafi AB, Ikhwanuddin M. (2023). Ovarian lavage method as an alternative route for hormonal administration and short-term sperm storage in *Clarias gariepinus*. Theriogenology 198: 203-209. https://doi.org/10.1016/j.theriogenology.2022.12.037

Putri NMSA, Pertami ND, Kartika GRA. (2021). Reproduction aspects of spotted barb (*Barbodes binotatus* Valenciennes, 1842) in Tamblingan lake. Jurnal Sumberdaya Akuatik Indopasifik 5(3): 303-310. https://doi.org/10.46252/jsai-fpik-unipa.2021.vol.5.no.3.159

Reid AJ, Carlson AK, Creed IF, Eliason EJ, Gell PA, Johnson PT, Cooke SJ. (2019). Emerging threats and persistent conservation challenges for freshwater biodiversity. Biological Reviews 94(3): 849-873. https://doi.org/10.1111/brv.12480

Roy A, Barui S, Banerjee R, Das BK. (2025). Hormonal regulation and disorder during fish disease. In: Das BK, Kumar V, editors, Laboratory techniques for fish disease diagnosis. Springer Nature Singapore. Pp. 419-439. https://doi.org/10.1007/978-981-96-4620-3_18

Sahadan FN, Christianus A, Ina-Salwany MY, Ismail FS, Othman R, Zulperi Z. (2022). Gonadotropin-releasing hormone – Its approaches to improve reproduction in fish. Sains Malaysiana 51(11): 3539-3549. https://doi.org/10.17576/jsm-2022-5111-03

Samarin AM, Żarski D, Palińska-Żarska K, Krejszeff S, Blecha M, Kucharczyk D, Policar T. (2017). In vitro storage of unfertilized eggs of Eurasian perch and its effect on egg viability rates and the occurrence of larval malformations. Animal 11(1): 78-83. https://doi.org/10.1017/S1751731116001361

Serrat A, Saborido-Rey F, Garcia-Fernandez C, Muñoz M, Lloret J, Thorsen A, Kjesbu OS. (2019). New insights in oocyte dynamics shed light on the complexities associated with fish reproductive strategies. Scientific Reports 9: 18411. https://doi.org/10.1038/s41598-019-54672-3

Sinjal H. (2014). Efektivitas Ovaprim™ terhadap ovulasi ikan lele. Budidaya Perairan 2(1): 14-21. https://doi.org/10.35800/bdp.2.1.2014.3788

Suryaningsih S, Sagi M, Kamiso HN, Hadisusanto S. (2012). Perubahan kadar hormon testosteron dan progesteron, korelasinya dengan indeks gonado somatik dan tingkat kematangan gonad pada ikan brek (*Puntius orphoides* Cuvier & Valenciennes, 1842). Biota: Jurnal Ilmiah Ilmu-Ilmu Hayati 17(1). https://doi.org/10.24002/biota.v17i1.129

Tang L, Xiao X, Shi H, Chen J, Han C, Huang H, Li S. (2020). Induction of oocyte maturation and changes in the biochemical composition, physiology and molecular biology of oocytes during maturation and hydration in the orange-spotted grouper (*Epinephelus coioides*). Aquaculture 522: 735115. https://doi.org/10.1016/j.aquaculture.2020.735115

Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levavi-Sivan B. (2003). Regulation of fish gonadotropins. International Review of Cytology 225: 131-185. https://doi.org/10.1016/S0074-7696(05)25004-0

Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O. (2010). Neuroendocrinology of reproduction in teleost fish. General and Comparative Endocrinology 165: 438-455. https://doi.org/10.1016/j.ygcen.2009.04.017

Citation

Amaliy I, Wulandari TE, Prastiya RA, Budi DS, Alghoul NM, Sasi SM. (2026). Short-term ovarian histological changes in response to combined Ovaprim™ treatment and intra-ovarian insemination in *Barbodes binotatus*. Letters in Animal Biology 06(1): 130 – 137. *https://doi.org/10.62310/liab.v6i1.387*