Technical Manual for Artificial Propagation of the Indonesian Catfish, *Pangasius djambal*
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Chapter IV

Induced Spawning

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The method of artificial breeding described in this chapter is presented in chronological order to facilitate the identification of the different steps of the breeding procedure. The involved operations require a minimum of rigour for successful spawning. This is even more important as broodfish have to be handled several times for selection, injections, ovulation checking and stripping.

SELECTION OF MATURE FISH

The first step is to select mature fish in the best condition from the cultivated broodstock in order to obtain the best quality of spawning.

General preparation and recommendation

Practical experience has shown that for selection of brooders, it is preferable to catch a small number of fish at the same time in order to minimize handling and stress. The use of index cards is necessary to record bio-data for every broodfish to increase the knowledge on their biology and to improve the technique.

Material necessary for the selection should be ready (Equipment and tools, Chapter III) prior start of fish capturing. The general handling precautions should be respected (see Chapter III).

After capture, in order to make the selection easier, each fish should be:

- slightly anesthetized;
- weighed;
- evaluated for their sexual maturity (biopsy for females or hand-pressure onto the abdomen for males).

Estimation of male sexual maturity is quickly done (Chapter III) and could be realized shortly after anesthesia and before weighing. Thereafter, ripe males can be isolated directly for reproduction while other fish are returned to their rearing structure.

Oocyte measurement and observation take a few minutes after intra-ovarian biopsy (Chapter III, Plate III.2). Mature females should be placed in a small private cage or pen, then recaptured only for injection. Non selected fish are released in their rearing structure until another checking.

Note on the *P. djambal* readiness (details in Chapter III)

- **Male readiness** is determined by production of milt at stripping using slight hand-pressure on the abdomen (scale 3).
- **Female readiness** is determined after intra-ovarian biopsy, by a homogeneous diameter distribution of oocytes sampled and a modal

diameter >1.7 mm. The bigger oocytes should be ivory and easy to separate from each other. The presence of a noticeable quantity of ovarian fluid in the biopsy is generally indicative of an ongoing process of oocyte resorption (atresia). External appearance (soft abdomen, swollen genital papilla, etc.) is not reliable enough to judge readiness in *P. djambal* females.

How many males per female?

Practically, the quantity of sperm collected from one male is generally sufficient to fertilize all ova collected from one or two females. However, producing a new fish generation from only one couple leads to a reduction in the genetic variability of the strain (consanguinity). This may result in a decrease of zootechnical performance after a few generations as already observed in several fish species including catfishes (Agnese *et al*., 1995).

P. djambal is a newly cultured fish with very good natural performance and excellent potential for aquaculture in Indonesia. If farmers cannot retain natural performance of this strain, the future production of this species is in jeopardy.

In fact, there are two targets for breeding fish, the first one is to constitute a new generation of brooders and the second one is to produce fry to be grown for human consumption. Even if these targets are different, farmers expect zootechnical performance at least as good as that of the parents.

Ideally, for maintenance of maximal genetic variability of new broodstock and prevention of reduced performance, it is recommended to use at least 10 males for 10 females, the sperm of each male being used to fertilize separately ova from each female (Gilles *et al.*, 2001). After hatching, an equivalent number of descendants from each crossbred should be reared together to constitute the future broodstock. However, even if this pattern of breeding is respected, the overall genetic variability of the strain will decrease slowly (around 5%) after each crossbreeding (Chevassus, 1989). This means that it should be necessary to introduce wild blood (wild fish) every 3 generations in order to ensure large genetic variability of the cultivated strain.

For each reproduction cycle of fish for grow-out, we recommend using the sperm pooled from 6 to 10 males for fertilizing ova collected from all the females induced (generally 2 to 4).

HORMONAL TREATMENT AND PROCEDURE

Once the most mature fish are selected, isolated in their pens and the other broodfish released into their rearing structure, the process of induced breeding can be started.

The term "mature" female means that oocyte growth has been achieved and that final oocyte maturation and ovulation can be induced through adequate hormonal stimulation. Various protocols of hormonal treatments were tested for triggering *P. djambal* ovulation (Legendre *et al.*, 2000a). So far the best quality of gametes has been obtained with the use of the following hormonal treatment (Legendre *et al.*, 2002).

Female

The hormonal treatment corresponds to two successive injections:

- one priming injection of hCG (*human chorionic gonadotropin*) at a dose of 500 IU (international unit) per kg female body weight. To prepare oocyte response to the ovulatory treatment (second injection). This priming injection never leads to ovulation by itself;
- one injection of Ovaprim (commercial mix of GnRh and Domperidone)[1] given 24-hours after the hCG injection, at a dose of 0.6 $mL.kg^{-1}$, for triggering ovulation.

Male

In order to increase the quantity of collected semen and reduce its viscosity, males received a single Ovaprim injection of 0.4 $mL.kg^{-1}$ given at the same time as Ovaprim injection of females.

Induced breeding procedure

If the previous recommendations have been applied fish should have already been weighed and isolated in a safe housing structure. That leaves time to calculate the right hormone quantity according to recommended doses, then to proceed to the first hormone injection.

Calculation of the hCG quantity

Product presentation

hCG is available in dehydrated powder form in sterile ampoules of 1500 and 5000 IU. These ampoules are presented in a small box together with 1-mL ampoules of 0.9% saline solution (Plate IV.1). The cost depends on the dollar rate. In March 2003, one ampoule of 1500 IU cost 76,400 IDR (Indonesian Rupiah) and 1 ampoule of 5000 IU, 144,567 IDR.

[1]) 1 mL of Ovaprim® (Syndel Laboratories, Canada) contains 20 µg of GnRha and 10 mg Domperidone.

Example of calculation

In the following example (Table IV.1), we take the case of two females ready to induce, of 4.5 and 6.5 kg body weight respectively. The first step in the induced breeding procedure is to calculate the quantity of hCG (in IU) to inject into each female.

Table IV.1.
Calculation of the quantity of hCG to inject.

	Body weight kg	hCG dosage IU.kg^{-1} of fish	hCG needed IU
Female 1	4.5	500	4.5 x 500 = 2250
Female 2	6.5	500	6.5 x 500 = 3250
Total	11	500	11 x 500 = 5500

Evaluation of the optimal quantity of hormone

To inject these two females in the most economical way, we have to take into account the number of hCG ampoules needed, because an ampoule once opened cannot be preserved for more than a few hours. In order to reduce the quantity of wasted hormone and thus optimize the operational cost, we compare several possibilities of ampoule combinations to reach the optimal ratio of cost to dose (Table IV.2).

Table IV.2.
Optimization between quantity and cost for hCG injection.

	Option 1	Option 2	Option 3	Option 4
Ampoule 1500 IU	3	0	4	1
Ampoule 5000 IU	0	1	0	1
Total hCG obtained (IU)	4500	5000	6000	6500
Correspondence in IU.Kg^{-1} of fish	409.1	454.6	545.5	590.9
	Far from wanted dosage	Near from wanted dosage	Near from wanted dosage	Far from wanted dosage
Total hormone cost* (IDR)	229,200	144,567	305,600	220,967

* In March 2003.

Calculation of the quantity to inject

From the calculation (in Table IV.2), it appears that the best option is to use one ampoule of 5000 IU hCG to inject both fish. This corresponds to the lower cost for a dose of 450 IU.kg^{-1}, still close from the recommended dosage for priming injection. The 5000 IU of hCG can be dissolved in 1 mL of 0.9% saline solution.

The following calculation shows the proper volume of hCG to inject into each female:

Table IV.3. Calculation of the volume of hCG solution to inject for each female.

	Body weight kg	Proportion of solution for each female	Corresponding volume mL	Round figure mL
Female 1	4.5	40.9% (4.5 / 11)	0.409	0.4
Female 2	6.5	59.1% (6.5 / 11)	0.591	0.6
Total	11	100%	1	1

As a general rule, the quantity of hCG to dissolve in 1 mL of saline solution should not exceed 5000 – 6000 IU. If the required quantity of hormone exceeds this amount, the volume of solvent should be increased accordingly.

Calculation of the Ovaprim quantity

Product presentation

Ovaprim is available in liquid form in sterile bottle of 10 mL. Its cost depends on the dollar rate. In March 2003, one bottle of this imported product cost 210,000 IDR.

Example of Calculation

In the following example, we prepare the second injection of the ovulatory treatment to the same females which already received the hCG priming:

Table IV.4. Calculation of the volume of Ovaprim to inject.

	Body weight kg	Ovaprim dosage mL.kg^{-1} of fish	Necessary Ovaprim mL
Female 1	4.5	0.6	4.5 x 0.6 = 2.7
Female 2	6.5	0.6	6.5 x 0.6 = 3.9
Total	11	0.6	11 x 0.6 = 6.6

The Ovaprim volume needed could be directly aspirated from the bottle with a sterilize syringe (Plate IV.2) and the remainder could be preserved in a cool place (refrigerator) for a few weeks.

Preparation of the injection

As hCG is available in powder for dissolving in 0.9% saline solution and Ovaprim is available as a liquid, it is clear that the process of preparation for injection of these two hormones is not the same. Details for specific preparation of each are presented in Plates IV.1 and IV.2.

Nevertheless, common rules have to be respected:

- in order to treat fish with accurate hormone doses, the size of the syringe used should be appropriate to the volume of liquid to inject. For example, 0.9 mL of hormone preparation should be injected with a 1-mL syringe and not with a 10-mL syringe;
- in order to prevent the solution from leaking out from fish body after injection, it is recommended;
  - to use the finest possible needle long enough to allow "deep" intramuscular injection. We recommend using a needle size of 0.70 x 38 mm.
  - To split the injection at different locations of dorsal musculature if the volume exceeds 1 mL for a small female (less than 4 – 5 kg body weight) or 2 mL for a bigger fish. In practice, it is better to prepare in advance the number of syringes needed in accordance to the volume to inject.
- Some days after induced breeding, skin and muscle necrosis is sometimes observed at the injection place. This results generally from infection brought by contaminated syringe or needle, or by products which have expired. In order to prevent such situation, it is strongly recommended to disinfect syringe and needle with alcohol before use or to use sterilized new materials for each series of induced spawning. It is also recommended to use a new bottle or ampoule of hormone each time.

Injection procedure

So far, no scientific comparison has shown a better ovulation or spawning when fish were injected intramuscularly or intraperitoneally. In practice either means of injection could be chosen, each with its own advantages and disadvantages (Harvey and Carolsfeld, 1993). The most important point remains that the total amount of injected hormone reaches the gonads, via blood flow, to trigger the ovulation process.

For *P. djambal*, we chose to deliver the hormone intramuscularly below the dorsal fin (Figure IV.1). At this place, the muscular mass is thick enough

and a deep injection could be done, limiting risk for the hormone preparation to leak out through the injection hole.

Handling for injection without anesthesia can be tolerated by the fish as far as it stays safely in its housing structure. In order to prevent stress (see detail in Chapter III), handle the fish with care, then wrap it gently in moist towel and maintain it in water. Only the dorsal part of the fish should emerge from the water in order to facilitate hormonal injection (Figure IV.1).

Injection should be done gradually. To help the liquid to find its way between muscle fibres, wait a few seconds before withdrawing the needle slowly.

After checking that there is no hormone leaking out through the injection hole, the fish is released in its housing structure, and then observed for a while to make sure that its behaviour is normal.

For females, these operations have to be repeated for the second injection.

Figure IV.1.
Hormonal injection on *P. djambal*.

FINAL MATURATION AND LATENCY TIME

The time lapse between hormone injection and ova collection is a key factor in the success of reproduction techniques involving hormone-induced ovulation and artificial fertilization in fish. In pangasiids, this latency period is defined more precisely as the delay between the second (last) injection and stripping of ova.

The aim of the first injection (hCG) is to prepare the gonad, increasing oocyte sensitivity to the second stage of hormonal treatment (Woynarovich and Horvath, 1980; Cacot *et al.*, 2002). This first injection generally results in a slight increase in oocyte diameter while germinal vesicle (nucleus) of oocytes remains in a central position. The process of final oocyte maturation and then ovulation is triggered entirely by the second injection (Ovaprim).

Following Ovaprim injection, the process of oocyte maturation includes migration of the germinal vesicle to the edge of the oocyte and germinal vesicle breakdown (GVBD). After GVBD, the oocyte is mature and ready for expulsion from the follicle (ovulation); then it becomes an ovum, ready for

fertilization. When the process of maturation is not complete, it is generally impossible to collect ova; eventually a few follicles could be obtained by hand stripping but are unable to be fertilized. For farmers, observation of the nucleus state and of the different phases of its migration allow better understanding of why spawning sometimes does not happen.

In order to observe nucleus position, a few dozen oocytes sampled by intraovarian biopsy can be fixed in Serra's fluid (30% formalin, 60% ethanol and 10% acetic acid in volume) for 5 to 15 minutes. After this period, oocytes become translucent and the nucleus is visible. Even if the nucleus can be seen using a magnifying glass, the use of binocular microscope (x 25) is recommended for accurate observation. The different stages of nucleus migration until ovulation are detailed in Plate IV.3.

Latency Time for *P. djambal*

In fish, delayed collection of gametes after ovulation leads to overripening of ova which can result in low fertilization rates, increase in the number of deformed embryos and lowering of embryo and larvae viability. The delay of ova survival varies according to species.

The process of overripening occurs rapidly in *P. hypophthalmus* (Legendre *et al.,* 2000b*)*. In order to obtain the highest egg quality in this species, the best period to collect ova is of short duration (no more than 2 hours) and occurs just after completion of ovulation.

In *P. djambal*, our observations indicate that a delay of 1 or 2-h after first ova occurrence (see infra) was necessary for collecting eggs of best quality, leading to highest fertilization and hatching rates.

The latency period between the last hormone injection and ovulation was negatively correlated with water temperature (Legendre *et al.,* 2002*)*. The higher the water temperature, the shorter is the latency period. Latency time to collect ova in *P. djambal* varied from 13 to 17 h for water temperature from 27 to 30°C (Table IV.5). It could be estimated by the following relationship: $LT = 20279\ WT^{-2.15}$, with LT, latency time, and WT, water temperature.

Water temperature (°C)	Latency time (h)
27	17
28	15
29	14
30	13

Table IV.5.

Latency time between second injection and ova collection as a function of water temperature in *Pangasius djambal.*

GAMETE COLLECTION AND PRESERVATION

In order to check ovulation and collect ova in good condition, all materials for fertilization and incubation should be ready to use. It has to be cleaned and prepared in advance.

Fish farmers generally use direct fertilization, which consists of stripping males and spreading milt directly over collected ova. This technique entails some risk of activating spermatozoa by urine, lowering their fertilizing ability before they could be properly mixed with ova. In the testis, spermatozoa are immobile. Their movements are initiated once semen is ejaculated and diluted in water. However in *P. djambal* the viability of sperm is of very short duration (about 30 seconds) and once spermatozoa stop moving they lose their fertilizing ability. To prevent this problem, milt should be properly collected and preserved.

Sperm collection and preservation

A delay of about 10 hours from hormonal treatment was sufficient to enhance spermiation in *P. djambal*. In practice, in order to give enough time for collecting sperm from 10 males before start of female checking, sperm collection should be start 9 to 10-h after hormonal injection, i.e. 2-h minimum before checking female ovulation for the first time.

Before starting the collection of milt, most of urine should be expelled from bladder by pressing gently the ventral area just anterior from the genital papilla. Then the fish papilla area and hands of manipulator should be dried (Figure IV.2) in order to prevent eventual mix of sperm with water.

Figure IV.2.
Papilla area is dried with absorbent paper before collection of sperm.

Sperm is collected by gentle squeeze of the abdomen as done for the assessment of maturity (Chapter III). In order to prevent activation of spermatozoa in case of mixing with some urine, the sperm is diluted immediately in an immobilizing solution (Cacot *et al.,* 2003). The most effective way to shorten the delay between sperm stripping and dilution is to

Figure IV.3.
Accurate measurement of the 0.9% saline solution.

Figure IV.4.
Sperm is aspirated directly into a syringe containing saline solution.

aspirate the milt directly into a syringe containing saline solution (0.9% NaCl; Figure IV.3 and IV.4).

The ratio of 1 volume of sperm to 4 volumes of saline solution is used. This dilution rate allows good preservation of sperm quality (fertilization ability) during a period of at least 24-h when dilute sperm is kept in a cool place at 4 – 5°C (refrigerator or cool box). As the sperm preparation is used most of the time for fertilization within a period of 2 to 6 hours after collection that generally ensures the availability of sperm of good quality.

After collecting sperm, each male can be released in its rearing structure until another reproduction cycle. The dilute sperm from all males is preserved in a cool place until fertilization.

How much sperm is it necessary to collect?

The total quantity of sperm needed for fertilization varies according to the total weight of ova collected, which is related to the body weight of induced females. About 1 mL pure sperm (5 mL diluted) is generally used for fertilizing 100 g of ova. So far, the quantity of ova collected from one *P. djambal* female after induced ovulation has not exceeded 10% of its body weight. This observation can serve to estimate the maximal volume of sperm needed in a given reproduction trial, as done in the following example.

If the total weight of induced females is 14 kg, maximal weight of ova that could be expected to collect is 1400 g (10% of female biomass). In order to have enough sperm, it is recommended to fertilize these ova with 70 mL of dilute sperm (5 mL per 100 g ova), i.e. 14 mL of pure sperm collected.

Assessment of stripping time, ova collection and preservation

Depending on water temperature (see Table IV.5), the checking for ovulation has to be started 11 to 12-h after the Ovaprim injection in order to collect ova

at the decisive moment. Checking should be repeated one to three times at 2-h interval when females have not ovulated yet.

As noted previously, females should be handled with care, then wrapped gently in a moist towel, covering the eyes and maintaining the fish in water. The belly has to be maintained out of the water in order to get direct access to the genital papilla.

To check the ovulation gentle hand-pressure on the abdomen towards the genital papilla has to be done carefully and assessment of stripping time is determined according to the following procedure:

- only ovarian fluid or ovarian fluid with about a dozen oocyte are extracted by gentle squeeze of the gonad indicates that the fish is not ready for stripping. The female is released in its housing structure for a supplementary period of 2 hours, until the second checking;
- more than 10 – 20 ova with no or very little ovarian fluid are extracted (Figure IV.6); the female is released for 1-hour and re-captured for direct stripping. It will be the **decisive stripping time**;
- emission of ova in the net without hand-pressure indicates that the female has to be stripped directly. The optimal stripping time is probably past already.

Figure IV.5.

Female requires careful handling for checking ovulation.

Figure IV.6.

Decisive stripping time.

Ova collection

Female should be taken out of the water carefully and the general handling precautions respected (Chapter III).

Once a female is in position for stripping, the papilla area and hands of the manipulator should be dried. If ova come into contact with water for some time, the micropilar canal will close and spermatozoa will not be able to penetrate the ova for fertilization.

After taking precautions, the stripping could be started and ova collected in a dry plastic bowl. Gentle hand-pressure is applied on the abdomen towards the genital papilla. The right time for stripping is characterized by a soft belly

and a continuous jet of ova at each hand-pressure (Figure IV.7). Generally, easy stripping leads to good quality of ova.

When ova are difficult to extract from the gonad and the female presents a rather hard belly, it is best to release the fish in the rearing structure.

Hard stripping generally leads to the collection of a dry mass of ova mixed with some blood (Figure IV.8); the hatching rate obtained from such eggs is generally very low.

Figure IV.7.
Easy stripping

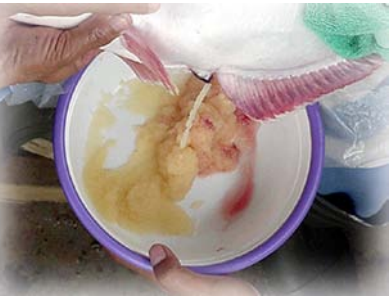

Figure IV.8.
Hard stripping

Our work experience has shown that excessive pressure on the belly could cause internal injuries and the fish could die.

However, when the stripping time is well evaluated and all the procedures described above respected, fatal situations occur rarely.

Before fertilization, collected ova could be preserved for more than 1-h when plastic bowl is covered, placed in shadow and protected against water splash. Indonesian farmers often immerse *P. hypophthalmus* ova in 0.9% saline solution for preservation before fertilization. However such practice should not be used for *P. djambal* ova. In fact, when placed in 0.9% NaCl solution for a few minutes, *P. djambal* ova could not be fertilized anymore, as was the case with freshwater.

To summarize, collected ova have to be preserved in a shady place without adding 0.9% NaCl solution and kept at a distance from any source of water.

EQUIPMENT AND TOOLS

Selection of mature fish

1. All equipment and tools listed in Chapter III should still be available.
2. The number of necessary small private cages or pens for isolating mature fish.

Hormonal treatment

1. Pocket calculator for calculating hormone doses.
2. Watch to note down injection time.
3. Thermometer for water temperature follows-up during latency period.
4. The necessary number of hCG ampoules or Ovaprim bottles.
5. Sterile ampoule or bottle of 0.9% saline solution (0.9% NaCl).
6. Sterile needle (size 0.70 x 38 mm).
7. Sterile syringe from 1 to 5 mL.
8. Alcohol for disinfecting reused needle or syringe.

Gamete collection and preservation

Males

1. Absorbent tissue paper for drying papilla area.
2. Bottle of sterile 0.9% saline solution.
3. Clean and dry syringe of 10 – 30 mL volume partly filled with saline solution for direct dilution of sperm during collection.
4. Plastic tubes with screw top for storing sperm preparation.
5. Small icebox with ice or refrigerator for preservation of sperm preparation.

Females

1. Absorbent tissue paper for drying papilla area.
2. Clean and dry plastic bowl for collecting ova.

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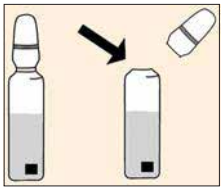
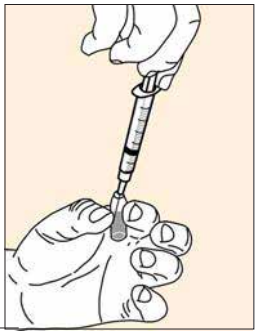
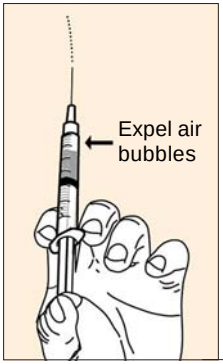


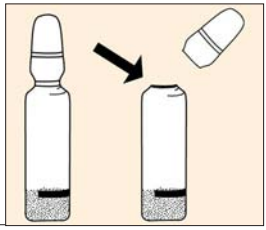


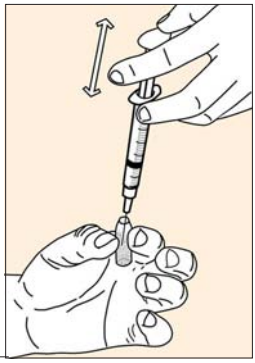
Open 1 ampoule of saline solution and collect the liquid in a 1 mL syringe.



Air bubbles should be expelled from the liquid then saline solution should be adjusted to 1 mL in the syringe using another ampoule of saline solution.

Open ampoule of hCG corresponding to the required quantity (see Table IV.2), then mix the saline solution with hCG powder, which is extremely soluble.

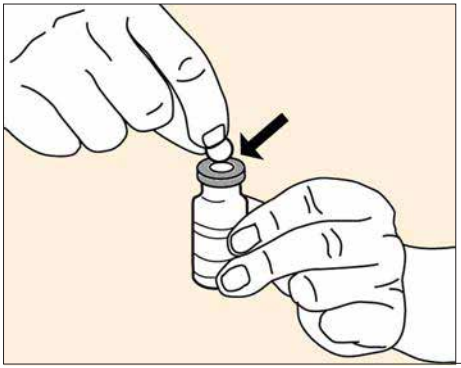




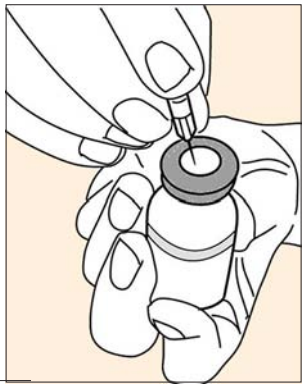
The 1 mL mixture of hCG and saline solution is delicately aspirated in 1 mL syringe. This hormone solution is ready to use, unless some air bubbles remain. In this case they should be expelled accurately with a minimum of hormone solution.

Plate IV. 1.

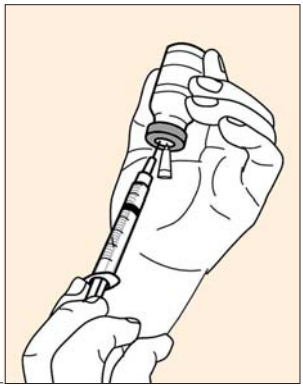
Procedure for hCG preparation.



Open 1 ampoule
of 10 mL Ovaprim.



Ovaprim liquid is thick.
To facilitate
the aspiration of
hormone,
it is recommended to
insert a second
needle through the top.

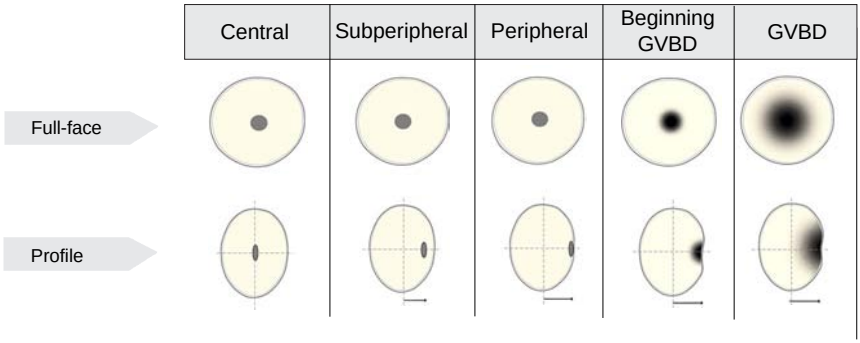


Necessary volume for one female is slowly aspirated in a 5 mL syringe. This hormone preparation is ready to use, unless some air bubbles remain.
In this case, they should be expelled again minimizing lost of hormone preparation.

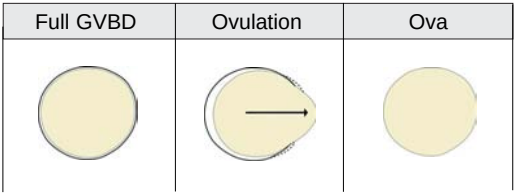
Plate IV. 2.

Procedure for Ovaprim preparation.

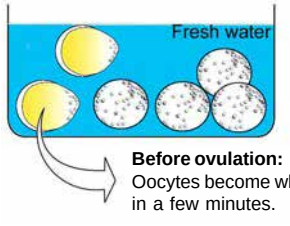
After 5 to 10 minutes in Serra's fluid the ova nucleus is visible. Then, it is possible to observe the difference stages of nucleus migration triggered by hormone treatment. Final oocyte maturation is described below.



Final oocyte maturation is followed by ovulation. After being released from their follicle, ova are ready to be collected and fertilized.

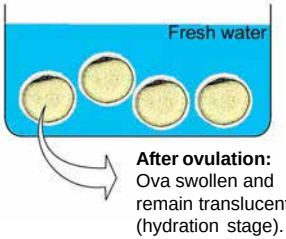


Determining the difference between oocytes in their follicle and ova with the naked eye is not so easy.



Before ovulation: Oocytes become white in a few minutes.

Practical advice: After immersion in fresh water the difference become clear.



After ovulation: Ova swollen and remain translucent (hydration stage).

Plate IV. 3.

Practical advice for observation of final oocyte maturation and for determining the difference between oocytes in their follicle and ova.

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Jakarta, 2003

DEPARTEMEN KELAUTAN DAN PERIKANAN

Title: Technical Manual For Artificial Propagation Of The Indonesian Catfish, *Pangasius djambal*

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Pictures: Jacques Slembrouck
Cover, layout and illustrations: Bambang Dwisusilo

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ISBN: 979-8186-92-3