PRINCIPLES OF FISH SEED PRODUCTION

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BACKGROUND

Induced fish breeding has been practised in Thailand since 1933, when the Thai Department of Fisheries successfully induced natural breeding in the common carp. In 1958, the technique of using pituitary hormones to induce spawning in fish was introduced to Thailand. This technique has been used effectively to induce spawning in several fish species: i.e., striped catfish, *Pangasianodon hypophthalmus* (Boonbrahm, 1959), walking catfish, *Clarias macrocephalus* (Tongsanga, 1961) and tawes, *Barbodes gonionotus* (Sidthiimunka, 1962). Recently, the technique of using gonadotropin releasing hormone analogues (GnRHAs), and a dopamine antagonist such as domperidone has been developed for fish species indigenous to Thailand (Sukumasavin and Leelapatra, 1988). This has proven to be very effective. Now, more than 20 species, which could not be effectively induced by pituitary gland extract, have been successfully induced to spawn using the GnRHA and domperidone approach.

BROODSTOCK MANAGEMENT

Teleosts are poikilothermic animals whose sexual maturity depends on water temperature. Under tropical conditions, most fishes become sexually mature within the first year (Horvath *et al.*, 1984). For example, *Barbodes gonionotus* matures sexually at the age of 8 months, when the length is only 8.5 cm (TL) and the weight 9 g (Paohorm, 1969). Although the fish is mature, the fertility of the eggs is poor. Thus, a low survival rate of the larvae has been reported from female *B. gonionotus* weighing 100 g. Therefore, the appropriate size of this species for breeding purposes is about 200 g (Joragun, 1978). In some tropical freshwater species, sexual maturation takes longer than one year: for example, 2 years in *Morulius chrysophekadion* (Unsrisong *et al.*, 1990), 3 years in *Osphronemus gouramy* (Pasukdee, personal communication), 4 years in *Probarbus jullieni* (Rodrarung *et al.*, 1990) and more than 10 years in *Pangasianodon gigas* (Sukumasavin, personal observation).

In hatchery operations, large broodstock are difficult to handle and require a longer period for adapting to captive conditions. Horvath *et al.* (1984) have suggested the following guidelines for the management of broodstock in captivity:

1. Select healthy fish with good physical characteristics.
2. Feed them with good quality food of the correct dietary composition.
3. Keep the broodstock at a low stocking density.
4. Identify the sex of the broodstock and keep separately if possible, because mixed stocks are inclined to spawn naturally.
5. Replace unspawned broodstock because the broodstock should not only tolerate but actually respond positively to induce spawning.
6. Keep spent (spawned) fish separately from the other broodstock and feed with protein-rich feed at 2-5% body weight per day, in order to promote recrudescence of eggs and sperms.
7. Produce natural feed by adding fertilizers regularly.
8. Select deep ponds for keeping broodstock and supply with adequate water, in order to ensure favourable water quality and to stimulate gonadal development.
9. Before each spawning season, add some trash fish into the feed, in order to promote gonadal development as well as recovery.
10. Stock new spawners with old spawners, for the replacement of broodstock.

FISH BREEDING

Fish reproduction is generally initiated by environmental factors (Fig.1 and Fig. 2), for example: temperature, rainfall, water quality, photoperiod, and food quality as well as food availability. The fish receives these signals through the brain and interprets them, in order to determine whether the environmental conditions are suitable for spawning. The message is then sent to the hypothalamus, which produces gonadotropin, releasing the hormone (GnRH). This is passed to the pituitary gland, which is located underneath the brain. The pituitary gland then produces gonadotropins (GtHs). Both GnRH and GtHs cause tiny incipient gonads to develop, mature and finally release gametes at the end of the process. This is a slow and long process, influenced by environmental temperature. In tropical countries, the process is faster than in temperate areas, where yolk formation slows down or almost stops during the winter season (Harvey and Carolsfeld, 1993).

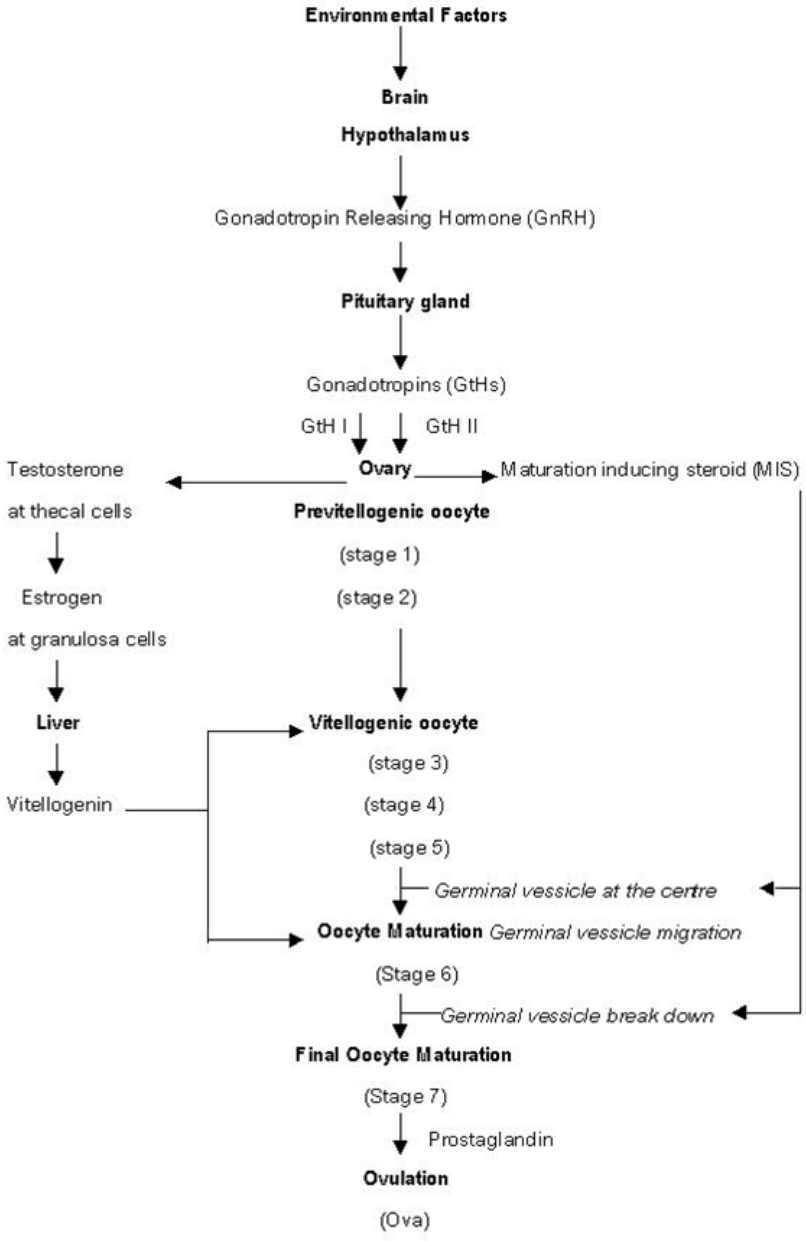


Figure 1: Events in the reproductive endocrine control of maturation and ovulation, amongst female teleosts

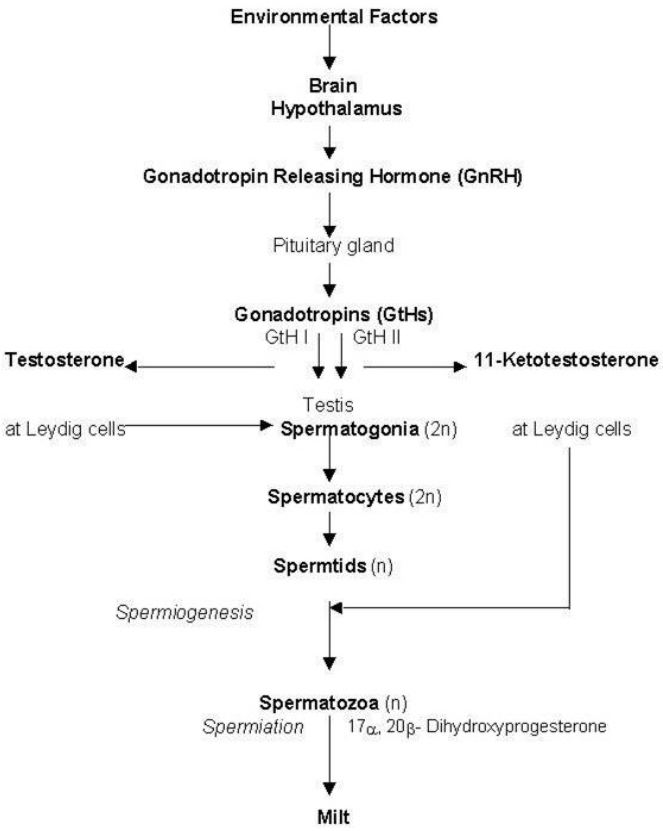


Figure 2: Events in the reproductive endocrine control of maturation and spermiation, amongstmale teleosts

Female gonadal development

In the female, GtH I stimulates steroidogenesis in the ovary to produce testosterone in the theca cells, where it is is then converted to estrogen by the granulosa cells of the oocyte follicle. The estrogen triggers the liver to start producing vitellogenin (yolk protein), which is sent via the blood and deposited in oocytes. The oocytes then develop slowly until mature. At that stage, the concentration of GtH I peaks. The ovary stops producing estrogen and instead starts producing a maturation-inducing steroid (MIS), under the control of GtH II. The MIS stimulates the germinal vesicle of the mature oocyte to migrate to the periphery of the oocyte and then break down at final oocyte maturation. Next, prostaglandin causes ovulation that result in free ova (Fig. 1). This process is called oogenesis and is similar in most teleosts. However, details may vary considerably in different species.

During oogenesis, oocytes are divided into various stages depending on the morphology of the nucleus, cytoplasm and follicle. These stages may be grouped into the previtellogenic, vitellogenic, maturation, and atresia phases. Details of each stage are completely documented in Selman and Wallace (1989).

Previtlellogenic phase: Oogonia undergo mitotic proliferation and enter the first meiotic division. The nucleus of the oocyte contains only one nucleolus (primary oocyte, stage 1). The primary oocyte is arrested in the ovary until it enters a growth phase (stage 2). The stage 2 oocyte increases in size progressively, surrounded by a layer of granulosa cells. The nucleus has several nucleoli at this stage, which are positioned in the peripheral region of the nucleus. RNA and mRNA of the oocytes are transported from the nucleus into the oocyte cytoplasm. They are called yolk nuclei or Balbiani bodies. The Balbiani bodies, which cause the oocyte cytoplasm to be stained basophilic, are initially located at the nuclear membrane (stage 2a) and subsequently migrate through the

periphery of the cytoplasm (stage 2b, 2c). During this growth phase, the volume of the oocyte increases about one thousand-fold. Ovaries in this stage contain immature oocytes and have a gonadosomatic index (GSI) of less than 2% (Selman and Wallace, 1989).

Vitellogenic phase: The oocytes increase in size due to endogenous and exogenous vitellogenesis. At the end of endogenous vitellogenesis, the Balbiani bodies disappear from the oocyte cytoplasm completely (stage 3). Next is a presentation of cortical alveoli at the oocyte cytoplasm (stage 4). The cortical alveoli are carbohydrate vesicles that stain for proteins and carbohydrates: for example; alcian blue, toluidine blue and periodic acid & Schiff reagents. The staining with the periodic acid & Schiff reagents results in pink cortical alveoli (stage 4) at the oocyte cytoplasm. However, the cortical alveoli are difficult to preserve. Thus, they loose their staining properties and assume a vacuolar appearance (Wallace *et al.*, 1987). Subsequently, the oocytes develop through exogenous vitellogenesis (stage 5), which is a long growth process under the control of pituitary gonadotropins. The enlargement of the oocytes is mainly due to accumulation of yolk that contributes 80-90% of egg dry weight (Wallace *et al.*, 1987). During stage 5, there is an increase of hepatic synthesis and a secretion of vitellogenin, lipophosphoprotein, and yolk protein precursor in response to the circulation of estrogen. The vitellogenin is delivered to the oocytes and forms yolk bodies by micropinocytosis. The yolk bodies accumulate in yolk globules and form yolk pellets. The nucleus of the stage 5 oocyte is reduced in size but still located at the centre of the oocyte.

Maturation phase: The nucleus of the vitellogenic oocytes starts migrating to the animal pole (germinal vesicle migration; GVM; stage 6). Next, the nuclear envelope of the oocyte breaks down (GVBD; stage 7). The oocytes have then reached the final maturation phase. The process of oocyte maturation is usually rapid and accomplished within 24 hours (Selman and Wallace, 1989). Finally, the mature oocytes are hydrated and released into the ovarian cavity (ovulation).

Atresia: Atresia is a degenerative process in which the oocytes at various stages stop growing and undergo rupture and resorption in situ by granulosa cell phagocytosis. Yolks are liquefied, forming irregular drops and staining acidophilic. Yolk residual may be seen as brown or black bodies. Atresia commonly occurs in fish ovaries during all development phases. In fact, there is no difference between the sizes of developing and atretic oocytes. Thus, fecundity estimates based on oocyte size may be affected by atresia (Macer, 1974). Female gonadal maturation may occur only once or else several times a year, depending on the species (reproductive cycle) and on environmental conditions (season, water and food).

Male gonadal development

Male gonadal development is called spermatogenesis. The process involves mitotic proliferation to produce large numbers of sex cells and meiotic division to generate genetic diversity and half the chromosome number. The process is initiated by environmental factors. The brain receives the signals and sends them to the hypothalamus. The hypothalamus releases GnRH to the pituitary gland, resulting in the production of GtHs. Initially, GtH I stimulates the Leydig cells of the testis to produce testosterone. The testosterone causes spermatogonia (2n) to undergo mitotic division to primary spermatocytes (2n). The primary spermatocyte enlarges in size and reduces the chromosome by half at the first meiosis, which results in secondary spermatocytes (n). The secondary spermatocytes then develop into spermatids at the end of the meiotic division. Next, GtH II peaks and stimulates the Leydig cells to produce 11-ketotestosterone, which induces spermatids to form a tail and become spermatozoa. The process is called spermatogenesis. The spermatozoa are the mature sperms in the lumen of the testis. A steroid called 17α, 20β-dihydroprogesterone, which stems from GtH II, causes the spermatozoa to dilute with seminal fluid and results in a sperm suspension called milt. This process is called spermiation. In nature, mature males with ripe gonads can be found over a longer period than mature females with ripe gonads. The spermatozoa may be ready for several weeks during the spawning season (Fig. 2).

Staging of gonadal development

The staging of gonadal development is necessary for propagation by hormonal administration. Effective hormone treatment requires the correct gonadal stage, which depends on the reproductive characteristics of the sex and species.

Staging of the female gonad

Gonadal development can be staged by various methods. Some methods may be quick and easy while other methods may be complex, expensive and time-consuming. Each method has some advantages as well as disadvantages. Female gonadal development can be staged in the following way.

External appearance of abdomen

In the female, a large, soft belly and swollen genital papilla indicates readiness for hormonal induction. External examination requires skill and experience for good results. Selection of broodstock based on these characteristics is fast, easy and causes little stress. However, the method is less useful for inexperienced farmers, and for the examination of new fish species, which might have different abdominal appearances.

Ovarian biopsy

Biopsy techniques can be used to sample eggs for staging by size (diameter) and appearance (color). The biopsy techniques are:

1) cannulating gonoduct with a fine polyethylene tube and aspirating a few dozen oocytes by mouth or by using a syringe. It is necessary to sample oocytes from the same part of the ovary in each fish to reduce sampling bias. This technique works in carps, catfish, milkfish, mullet, rabbitfish, seabass and grouper etc (Harvey and Carolsfeld, 1993).
2) puncturing the abdominal wall with a hypodermic needle and withdrawing some oocytes. The technique is effective and quick in less ripe gonads and in some species with fragile oviducts. The technique is less damaging than cannulation.
3) incising the abdominal wall and removing ovarian tissue. The technique is applied to large fish where the other biopsy techniques do not work. The sampled oocytes are cleared with clearing solution (40% formalin, 40% ethanol and 20% glacial acetic acid) to find the position of the germinal vesicle or nucleus of the oocytes, which may be classified into central (stages 2-5), migrating (stage 6) and peripheral (stage 7) germinal vesicles. Readiness for hormonal induction is indicated when one third of the oocytes have germinal vesicles in migrated and peripheral stages, while the rest have a central nucleus. Using clearing solution, it takes 1-2 minutes to clear up the oocyte yolk. The nucleus will only be visible for 4-5 minutes (Harvey and Carolsfeld, 1993).

Oocyte diameter and distribution

The diameter of the oocytes can be measured by expelling the sample into a fixative solution of 5% phosphate-buffered formalin placed in a petri-dish. Using a microscope with an ocular micrometer, about 50 oocytes are measured. The average oocyte diameter as well as the size distribution can be used to predict the appropriate timing for hormonal induction, when compared to a reference. In milkfish for example, an average oocyte diameter of 750μm or more is the critical size for GnRHa treatment. Diameter distribution is useful in species where the diameter of the oocyte does not significantly increase after the completion of vitellogenesis (Harvey and Carolfeld, 1993).

Ovary weight and oocyte morphology

The technique needs a representative sample of the broodstock. Thus, a few fish (3-5) are sacrificed and ovaries dissected. The ovaries are weighed for calculation of the gonadosomatic index (GSI (%) = ovary weight/body weight x 100) and then processed for histological sectioning.

The GSI can be used to predict the gonadal stages of the fish, when compared to a reference. The histo-morphological structure of oocytes in the ovary is the most accurate method for oocyte staging. However, there are many oocytes in an ovary. Thus, the average oocyte stage is required to decide the ovarian stage. The stereological method is based on the Delesse Principle, which assumes that a random 2-dimentional section (histological slide) can be used to quantify the composition of a 3-dimentional object (ovary). Thus, the stereological method presents a composition of the ovary, which is occupied by oocytes at each stage. Although the method is effective, it requires expensive equipment, knowledge of stereology and is time-consuming. The method has been used to quantify ovarian composition and the maturity of some aquatic animals (Lowe *et al.*, 1982; MacDonald and Thompson, 1986).

Staging of the male gonad

The staging of the male gonad is simpler and easier than the female. The ripe male can be staged in the following ways.

Secondary sexual characteristics

Mature males show secondary sexual characteristics such as:

1) pearl organs on the bodies and pectoral fins of cyprinids,
2) distinct body colors, like dark and bright colors on the chins of tilapias or black spots on the pectoral fins of Giant gourami (Srisakultiew et al. 1994),
3) protruding genital papillae in *Clarias* spp.

Spermiating male

Males generally produce milt in captivity. The spermiating male has sperm in the lumen of the testis and sperm duct. During the final stage of maturation in the male (hydration), seminal fluid is released in the lumen. The volume of sperm increases dramatically, and sperm become more fluid and diluted. The male may remain in this condition for several months without spawning (Harvey and Carolsfeld, 1993). Ripe males are easily distinguished by the release of milt, when the belly is pressed or squeezed.

Breeding Techniques

Some cultured species spawn in captivity easily but some may not spawn at all. For those that do spawn, it may be asynchronously, or in a season not necessarily desired by the farmer for optimal management. Thus, breeding techniques play an important role for large-scale fry production. Breeding techniques can be divided into 2 categories.

Natural spawning

Males and females are released together in a spawning pond where external cues are manipulated, (e.g. temperature, water exchange, water quality, photoperiod and presence of the nest), to stimulate the fish into spawning naturally. The spawners may be left to incubate their eggs, or else they may be separated, depending on their brooding behaviour. In Thailand, there are approximately 11 species where natural spawning is used (Table 1). This technique is also called uncontrolled breeding or semi-controlled breeding.

Table 1. Freshwater species that are reproduced by natural spawning

Common name	Scientific name	References
Giant gourami	*Osphronemus gouramy*	Srisakultiew *et al.*, 1994
Nile tilapia	*Oreochromis niloticus*	Pongsuwan and Sithimungka,1989
Catfish	*Clarias macrocephalus*	Pongsuwan and Sithimungka,1989
Catfish	*Clarias batrachus*	Pongsuwan and Sithimungka,1989
Royal featherback	*Chitala blanci*	Supachalust, 1988
Spotted Featherback	*Chitala ornata*	Rodrarung and Meewan,1996
Swamp eel	*Monopterus albus*	Kwanmuang *et al.*, 1993
Freshwater Garfish	*Xenentodon cancila*	Juntubtim, 1996
Drumfish	*Boesemania microlepis*	Pimolbutr and Pasugdee, 1994
Sand Goby	*Oxyeleotris marmorata*	Amatayakul *et al.*, 1995

Hormonally induced spawning

A hormone is administered, in order to stimulate the fish to spawn either naturally or artificially. This method is usually applied to cultured fish that have matured but lack the ability to spawn in captivity. However, the technique has become routine practice on fish farms because it is relatively easy, efficient and practical. The hormones used for manipulating fish maturation and ovulation include pituitary extract, HCG, and GnRH/LHRH plus their analogues, in combination with dopamine antagonist. Details of the hormones used in aquaculture are explained below.

Fish pituitary extract (hypophysation)

This method was established in 1931 by Houssay, who used fish pituitary extract or hypophysation to induce fish spawning (Harvey and Hoar, 1979). Fish pituitary glands are obtained from sexually maturing or mature donors either of the same or of a different species. The gland may be used fresh or stored in absolute alcohol or acetone. General computation of the hypophysation working dose is based on the fresh weights of the donor and the recipient:

$$\text{Working Dose} = \frac{\text{Weight of Donor Fish (kg)}}{\text{Weight of Recipient fish (kg)}}$$

Fish are induced to spawn by administering the working dose once or twice (Table 2, p 16). For example, a single injection, of 1.4-2.0 times the working dose of the hypophysation, induced *B. gonionotus* to spawn 4-6 hours after administration (Tavarutmaneegul *et al.*, 1992). This method is practical in the field, because simple equipment and small amounts of materials are needed. The whole pituitary gland contains other pituitary hormones in addition to the gonadotropins, which may increase the efficacy of hypophysation (Donaldson, 1986).

The method however, has some disadvantages:

- Many donor fish have to be killed to obtain pituitary glands.
- The pituitary extract is unreliable, due to poor standardization.
- In China, there is some evidence that fish may develop an immune reaction to repeated injections.
- When fresh pituitary extract is used, the donor fish may transmit a disease to the recipient.
- The weight of the donor fish may be unknown, when stored or dried pituitary glands are used.

Using dried pituitary glands, Rowland (1983) reported that a very low dose (1 mg. carp pituitary gland per kg. of the recipient) induced oocyte maturation without ovulation in the golden perch (*Macquaria ambigua*), whereas a somewhat higher dose (5 mg/kg) induced 100% ovulation. However, the fertilization rate was more variable at 5 mg/kg than at the relatively high dose of 10 mg/kg. On the other hand, a really high dose (15 mg/kg recipient) reduced hatchability when compared to 10 mg/kg, which appeared to be the optimal dosage for the species.

Although the method solves the problem of the unknown weight of the donor, it requires a fine electrical balance for weighing the gland to the nearest milligram. The technique has been improved by using a lyophilised pituitary powder, which is a crude preparation of fish gonadotropin. This powder is more stable, as well as having a longer shelf life and known potency of the gonadotropin content, than the fresh pituitary gland of either a carp or a salmon (Yaron and Levavi-Zermonsky, 1986). Induced spawning with a lyophilised pituitary gland is quite expensive and only efficient when used with closely related species. Furthermore, the hypophysation approach may later face problems as new fish species are introduced to aquaculture, and closely-related fish pituitaries are difficult to obtain (Yaron and Zohar, 1993)

Human Chorionic Gonadotropin

Human Chorionic Gonadotropin (HCG) is extracted from the urine of pregnant women. This gonadotropin is a complicated glycoprotein, with a molecular weight of about 30,000 Dalton. It is uniform in a given batch and can therefore be standardized. HCG is also available as a pharmaceutical product. The use of HCG eliminates the need for killing fish and the whole pituitary preserving process (Donaldson, 1986). However it works in some fish gonadotropin receptors, but not in others. Sometimes, the HCG may work on the male but not the female. In addition, the effective dosage of HCG may vary from species to species, depending on how closely related the fish endogenous gonadotropin is to HCG (Lam, 1982). Nonetheless, HCG alone, or in combination with hypophysation, has efficiently induced ovulation in a number of fish species. The method is common practice on many fish farms, despite the fact that HCG is a relatively expensive product.

Gonadotropin-Releasing Hormone, or luteinizing hormone-releasing hormone, their analogues and dopamine antagonists

The gonadotropin-releasing hormone (GnRH), or the luteinizing hormone which releases another hormone (LHRH), is a short peptide hormone, composed of 10 amino-acids. The peptide is similar in most teleosts and mammals (Donaldson, 1986). Analogues (GnRHa or LHRHa) are molecules in which some amino-acids have been substituted, in order to increase the half-life of the molecule in the fish and so increase its capacity to bind with GnRH receptors in the pituitary gland. Thus, the efficacy of the GnRHa/LHRHa will be higher than that of natural forms of the GnRH/LHRH. The use of either GnRH/LHRH or GnRHa/LHRHa for spawning induction has several advantages over the traditional hypophysation technique. The GnRH/LHRH or GnRHa/LHRHa is a small peptide molecule. Thus, it can be synthesized into its native form and also into altered forms (analogues), with a slow rate of degradation. Therefore, lower doses are required when using analogue forms (Zohar *et al.*, 1989). GnRH/LHRH and its superactive analogues (GnRHa/LHRHa) are unlikely to elicit immunological responses, like some heterologous gonadotropins (hypophysation and HCG) do, and are therefore potentially less harmful to the recipient fish. In addition, GnRH/LHRH (GnRHa/LHRHa) stimulates the secretion of the fish's own GtHs. This means that it is not species-specific and can be successfully applied to a great variety of fish species.

Studies on the brain's regulation of hypophysial function by Peter *et al.* (1986) revealed the presence of two hypothalamic hormones that control the release of GtHs from the pituitary gland. The first is GnRH, which stimulates GtHs release. The second is dopamine, an amine which antagonises the release of the GnRH in most teleosts, and thus reduces the production of GtHs from the pituitary gland. Therefore, injecting either GnRH/LHRH or GnRHa/LHRHa alone is generally ineffective in inducing ovulation in cultured fish, due to the strong inhibitory effect of dopamine on GtHs secretion. This has been found to be the case with goldfish, common carp and Chinese carps (Peter *et al.*, 1986; Lin *et al.*, 1986). Lin and Peter (Peter *et al.*, 1988) developed the "Linpe method", by injecting a combination of a GnRHa/LHRHa and a dopamine antagonist (domperidone, pimozide or metoclopramide), in order to induce the ovulation and spawning of cultured fish. The Linpe method has been widely and effectively applied in many cultured fishes throughout the world (Lin and Peter, 1996). In Thailand, the Linpe method has also been used

successfully in many fish species, as shown in Table 2, p.16. However, it is important to note that the dopamine antagonists vary in their efficacy amongst different fish species (Zohar, 1988; 1989).

In some cases, GnRHa is used in combination with carp pituitary extract or HCG, in order to induce the ovulation and spawning of cultured fish species (Peter et al., 1988a; Table 2). In addition, the sustained release of GnRH from slow-releasing vehicles (cholesterol, cholesterol-cellulose or biodegradable polymers), has been successfully used to induce continuously high GtHs levels and/or ovulation in salmonids (Crim and Glebe, 1984), goldfish (Sokolowska et al., 1984), milkfish (Marte et al., 1987), sea bass (Almendras et al., 1988) and gilthead sea bream (Zohar, 1988). The rate of GnRH release can be determined by altering the proportion of cellulose in the cholesterol pellet (Sherwood et al., 1988).

Embryogenesis

Many fish species require that their embryos (eggs) incubate and hatch in open water. Eggs are broadcast in the water column and either float or sink; adhesive eggs may attach to plants or hard substrates (rock or gravel). Eggs from other fish are laid in a nest, and parent(s) provide a constant water flow by fanning their fins. Some fish also incubate eggs in their mouths where movement of the gill plates provides both gentle tumbling and water circulation. Artificial incubation and hatching of fish embryos simulate these natural processes. In the wild, eggs (or egg masses) are susceptible to predation, and are easily damaged by the continual change of the natural environment. The advantage of man-made hatcheries is that the environment can be controlled and manipulated.

Developing embryos and newly-hatched larvae (fry) are the most sensitive and delicate of the stages in the life history of a fish. Therefore, great care must be taken to provide them with the proper incubating and hatching environment. Water temperature, light, water quality, water flow, shock prevention, and type and size of the egg are very important considerations.

Embryogenesis is the process by which the embryo is formed and develops. It starts with the fertilization of the ovum, egg, which, after fertilization, is then called a zygote. The zygote undergoes rapid mitotic divisions, the formation of two exact genetic replicates of the original cell, with no significant growth (a process known as cleavage) and cellular differentiation, leading to development of an embryo. It occurs in both animal and plant development, but this article addresses the common features among different animals.

The zygote: The egg cell (and hence the fertilized egg) is always asymmetric, having an "animal pole" (future ectoderm and mesoderm), two of three primative tissue types, and a "vegetal pole" (future endoderm), it is also covered with different protective envelopes. The first envelope, the one which is in contact with the membrane of the egg, is made of glycoproteins and is called vitelline membrane. Different taxa show different cellular and acellular envelopes.

Cleavage: The zygote undergoes rapid cell divisions with no significant growth, producing a cluster of cells that is the same size as the original zygote. The different cells derived from cleavage, up to the blastula stage, are called blastomeres. Depending mostly on the amount of yolk in the egg, the cleavage can be holoblastic (total) or meroblastic (partial).

2-cell stage: The first cleavage furrow, ending the first zygotic cell cycle, is vertically oriented, as is usual until the 32-cell stage. The furrow arises near the animal pole and progresses rapidly towards the vegetal pole, passing through only the blastodisc and not the yolky region of the egg. Near the bottom of the blastodisc the furrow changes to a horizontal orientation to undercut the blastodisc, but still leaves the cells only partly cleaved from the underlying yolky region. The two blastomeres are of equal size and appear otherwise undistinguished from one another. The following several cleavages are strictly oriented relative to the first one. However, the eventual

axes of body symmetry (i.e. the dorsal-ventral and anterior-posterior axes) apparently cannot be predicted with any certainty from the orientation of the cleavage.

4-cell stage: The two blastomeres cleave incompletely and in a single plane that passes through the animal pole at right angles to the plane of the first cleavage. Hence, cycle 3 begins with 4 blastomeres in a 2x2 array. A view from the animal pole reveals that the blastodisc is ellipsoidal in shape. The second cleavage plane is oriented along the longer axis.

8-cell stage: Cleavages ending cycle 3, still incomplete, occur in two separate planes, parallel to the first one, and on either side of it. They cut the blastodisc into a 2x4 array of blastomeres. As the dechorionated embryo usually lies in a dish, the 4-cell aspect, rather than the 2-cell aspect, faces the observer. This "face" view is along the odd numbered cleavage planes (furrows 1 and 3 are visible;). The dechorionated embryo tends to lie in the same orientation through late blastula stages (through the high stage).

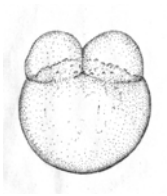
16-cell stage: The fourth set of cleavages also occur along two planes, parallel to and on either side of the second one, and produces a 4x4 array of cells. Use care to distinguish this stage from the 8-cell stage, because they look similar in face. For the first time some of the cells now become completely cleaved from the others. These 'complete' cells are the 4 most central blastomeres, the quartet that is entirely surrounded by other cells. Their complete cleavage occurs near the end of the 16-cell stage because of the way the cleavage furrows undercut the blastodisc from the center, going outwards towards the blastodisc margin. Indeed, the undercutting furrows still do not reach the margin, and the 12 cells surrounding these 4 central ones, the so-called marginal blastomeres, remain connected to the yolk cell by cytoplasmic bridges. From this stage onwards until the midblastula period the cleavages completely partition most or all of the nonmarginal blastomeres, but still incompletely partition the marginal ones.

32-cell stage: The cleavages ending cycle 5 often occur along four parallel planes, rather than two, lying between those of the first and third cycles. However, oblique orientations of the furrows are now common. Frequently the 32 blastomeres of this stage are present in a 4x8 array, but other regular patterns, as well as irregular ones involving one or more of the blastomeres also occur. In a side view one usually sees two **tiers**, or horizontal rows, of blastomeres between the margin and the animal pole. This is because the plane of the blastodisc is curved; marginal cells are more vegetal, and they lie partly in front of the nonmarginal ones positioned closer to the animal pole.

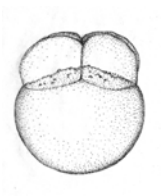
64-cell stage: Cleavages ending the sixth cycle pass horizontally, so that in an animal polar view the blastomere array may look similar to the 32-cell stage, although the cells entering cycle 7 are smaller. From the side the cell mound looks distinctly higher. For the first time some of the blastomeres completely cover other ones. The buried cells, or deep cells, each arise as one of the two daughters of the 4 central blastomeres that were present at the 32-cell stage. The other daughter remains superficial, in the top-most tier of what is now the enveloping layer (EVL) of the blastodisc. During the same cleavage the horizontal divisions of marginal blastomeres present at cycle 6 produce two EVL sister cells, and in a face view of the 64-cell stage one sees three tiers of EVL cells.

Morula The morula is produced by embryonic cleavage, the rapid division of the zygote. After reaching the 16-cell stage, the cells of the morula differentiate. The inner blastomeres will become the inner cell mass and the blastomeres on the surface will later flatten to form the trophoblast. As this process begins, the blastomeres change their shape and tightly align themselves against each other to form a compact ball of cells. This is called compaction and is likely mediated by cell surface adhesion glycoproteins.

Zygote



2 cell

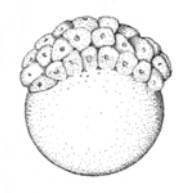


4 cell

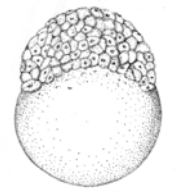
8 cell

16 cell

32 cell

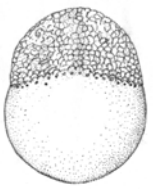


64 cell

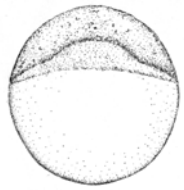


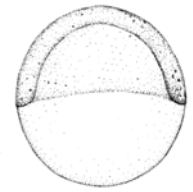
Early blastula

Morula stage

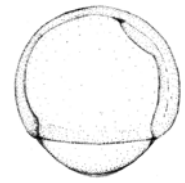


Late blastula

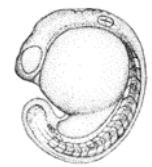


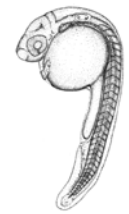


Gastrula stage

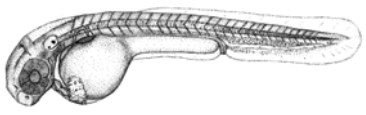


Organogenesis

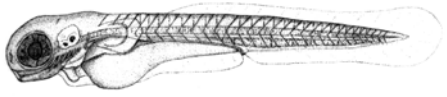




Morphogenesis



Hatch Out



Larva or Fry

Blastula and Gastrula After the cleavage has produced over 100 cells, the embryo is called a blastula.The blastula is usually a spherical layer of cells (the blastoderm) surrounding a fluid-filled or yolk-filled cavity (the blastocoel)**.**

During **gastrulation** cells migrate to the interior of the blastula, consequently forming two (in diploblastic animals) or three (triploblastic) germ layers. The embryo during this process is called a **gastrula**. The germ layers are referred to as the ectoderm, mesoderm and endoderm. In diploblastic animals only the ectoderm and the endoderm are present.

The first stage of gastrulation begins with the epiboly of the EVL and the deep cells over the YSL. This epiboly is driven by the migration of nuclei and cytoplasm in the YSL and attachments between the YSL and the EVL. Intercalation of the deep cells with the EVL help drive this movement. At about 50% of epiboly, a fate map similar to that of the Xenopus can be derived. The EVL develops into an extraembryonic membrane and does not contribute to the embryo.

The second stage of gastrulation occurs when the leading edge of the epibolizing blastoderm thickens. The dorsal side forms a larger thickening and is known as the **embryonic shield**. The deep cells in the embryonic shield form two layers. The **epiblast** forms near the surface and will give rise to the ectoderm. The **hypoblast** forms next to the YSL and will form a mixture of endoderm and mesoderm. The hypoblast is formed through involution and/or ingression. The movement of cells in the hypoblast are similar to the involuting mesoderm of amphibians. The end result of gastrulation is an asymmetric involution of cells that form the dorsal structures of the embryo.The following processes occur to place the cells in the interior of the embryo.**Epiboly** - expansion of one cell sheet over other cells. **Ingression** -cells move with pseudopods.**Invagination** - forming the mouth, anus, and archenteron**.Delamination** - the external cells divide, leaving the daughter cells in the cavity.**Polar proliferation**

Other major changes during gastrulation: Heavy RNA transcription using embryonic genes; up to this point the RNAs, Used were maternal (stored in the unfertilized egg). Cell start major differentiation processes, losing their pluripotentiality.

Organogenesis is the process by which the ectoderm, endoderm, and mesoderm develop into the internal organs of the organism. The germ layers in organogenesis differ by three processes: folds, splits, and condensation. At some point after the different germ layers are defined, **organogenesis** begins. The first stage in vertebrates is called **neurulation**, where the neural plate folds forming the neural tube. Other common organs or structures which arise at this time include the heart and somites

The proceeding graph represents the products produced by the three germ layers.

Germ Layer	**Category**	**Product**
Endoderm	General	Gastrointestinal tract
Endodern	General	Respiratory tract
Endoderm	General	Endocrine glands and organs (liver and pancreas)
Mesoderm	General	Bones
Mesoderm	General	Most of the Circulatory system
Mesoderm	General	Connective tissues of the gut and integuments
Mesoderm	General	Excretory Tract
Mesoderm	General	Mesenchyme
Mesoderm	General	Mesothelium
Mesoderm	General	Muscles
Mesoderm	General	Peritoneum
Mesoderm	General	Reproductive System

Mesoderm	General	Urinary System
Mesoderm	Vertebrate	Chordamesoderm
Mesoderm	Vertebrate	Paraxial mesoderm
Mesoderm	Vertebrate	Intermediate mesoderm
Mesoderm	Vertebrate	Lateral plate mesoderm
Ectoderm	General	Nervous system
Ectoderm	General	Outer part of integument
Ectoderm	Vertebrate	Skin (along with glands, hair, nails)
Ectoderm	Vertebrate	Epithelium of the mouth and nasal cavity
Ectoderm	Vertebrate	Lens and cornea of the eye
Ectoderm	Vertebrate	Melanocytes
Ectoderm	Vertebrate	Peripheral nervous system
Ectoderm	Vertebrate	Facial cartilage
Ectoderm	Vertebrate	Dentin (in teeth)
Ectoderm	Vertebrate	Brain (rhombencephalon, mesencephalon and prosencephalon)
Ectoderm	Vertebrate	Spinal cord and motor neurons
Ectoderm	Vertebrate	Retina
Ectoderm	Vertebrate	Posterior pituitary

In most animals organogenesis along with **morphogenesis** will result in a larva. The hatching of the larva, which must then undergo metamorphosis, marks the end of embryonic development.

Morphogenesis is one of three fundamental aspects of developmental biology along with the control of cell growth and cellular differentiation. Morphogenesis is concerned with the shapes of tissues, organs and entire organisms and the positions of the various specialized cell types. Cell growth and differentiation can take place in cell culture or inside of tumor cell masses without the normal morphogenesis that is seen in an intact organism.

Egg Incubation

Artificial incubation of fish eggs is a hatchery practice that will increase the economic efficiency of a commercial fish culture operation. Hatching rates and survival will be increased using artificial incubation. Also, removal of the eggs from the parents may increase egg production by shortening the time for another spawning to occur.

Proper Incubation of Fish Eggs

Fish embryos and larvae are susceptible to many types of organic or inorganic materials dissolved or suspended in the water. These may include gases, minerals, metals, and particulate matter from rocks, soil, plants and animals. It is essential to know the water quality standards for embryos and larvae of the particular fish species. General water quality standards used in fish culture can be used as a reference point for hatchery water.

Hatching temperature and dissolved oxygen:

Spawning of brood stock, embryo development, survival, and growth of fish larvae occur within a narrow range of water temperatures. Incubation temperature has a direct effect on the timing of embryonic development and thus determines hatch rate. Fish development and hatching is delayed at low temperatures, and accelerated at high temperatures. Incubating temperatures are also known to modify the behavior of larvae and determine certain morphological characteristics. There is an optimum temperature required for each developmental life stage, and these vary among species. Water temperatures should be maintained with minimal fluctuations, preferably no more than ± 1°C (2°F) from optimal. If a species' optimum water temperature for incubation is unknown, use the optimum temperature of a related species or of a fish that inhabits a similar geographic area. In general, optimum temperatures for spawning, incubating, and rearing newly-hatched tropical freshwater species are 24-28°C (75-82°F). Avoid temperatures above or below this range. Poor embryo survival, low hatch success, reduced growth rates, larval deformities, and increase in

fry/larvae diseases often result from temperature fluctuations or temperatures outside the optimum range for the species. Take the embryo development of Silver carp as an example, when the water temperature is 18°C, the incubating process takes 61 hours; when the water temperature is 28°C, only 18 hours are needed.
Dissolved oxygen content in pond water should not be lower than 4--5mg/l. Below 2mg O_2/L, the embryo can not develop normally.

The amount and incidence of light received during incubation can affect both fish development and larval survival. Incubation of fish embryos should occur in either dim light or darkness. Light can also be used to synchronize hatching. Many species of fish will not hatch in daylight, therefore, if the lights are switched off, hatching will occur a few hours later.

During incubation, a constant water flow is essential for preventing accumulation of waste products and allowing gas exchange between the egg and the surrounding water. Constant motion also appears to be necessary for successful hatching for some species of fish. Proper water flow also reduces mechanical abrasion. Eggs of many fish are sensitive to mechanical shock and should not be moved during certain times during development. For example, eggs of salmon and trout can only be moved during the first 36 hours after fertilization. Thereafter, the eggs are kept still until the embryo eye becomes visible. The amount of water flow necessary for proper incubation of fish embryos depends largely on egg density (how heavy and large eggs are in water). Some fish eggs are quite dense and sink to the bottom when released. Other eggs become buoyant as they "water-harden" and free-float in the water column or at the surface. Some eggs have hair-like structures or specialized coatings that make them sticky. Some eggs have an oil drop in them and they float on the surface.

Egg diameter is also an important consideration during incubation. Screen mesh size should prevent the passage of eggs while allowing sufficient water circulation and detering debris collection. Most ornamental fish eggs are around 0.8 mm in diameter, however, the size range is wide. Eggs can be as large as 1.5-2.0 mm for some ornamental catfish, and as small as 0.4 mm for gobies.

Types of Fish Egg Incubators
A wide variety of devices are used for incubating fish eggs. For practical purposes, we have classified fish egg incubators into three major types: egg mats, trays, and conical incubators. Their use is based primarily on the density of the eggs to be hatched, their stickiness, and the sensitivity of the eggs to mechanical shock. Figure 1, Figure 2, and Figure 3 illustrate the three general types of fish egg incubators.

Egg mats are used primarily for adhesive eggs. By simulating a spawning substrate (plants, rocks, etc.), they serve as egg collectors and provide a place for egg attachment. Since egg mats also serve as a stimulus for spawning, they are also known as spawning mats. Mats consist of bundles of fibrous material arranged in a variety of forms and made from a variety of different materials (plastic shreds, air filters, spanish moss, coconut fibers, horse hairs, etc.) (Figure l, a and b). Typically, egg mats are suspended in the water column or laid along the bottom or sides of the spawning container. The mats can be removed from the spawning container and suspended in the air where they can be kept moist at all times with a fine spray of water. The oxygen content of air is about 20 times more than water, thus increasing gas exchange between the egg and the thin film of water that surrounds them. For spawning and incubating eggs of many ornamental fish, such as angelfish, discus, and corydoras catfish, mats are often replaced with bottle brushes, pots, or slates that are made of plastic, glass, clay or rock (Figure 1c).

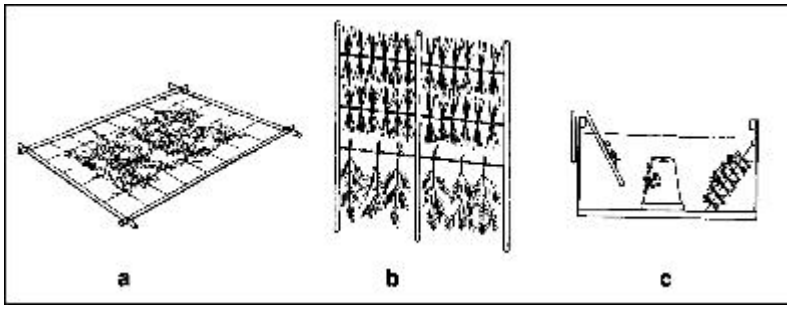


Figure 1. Egg mats: frameworks with a) spanish moss and b) bundled fibers; c) slate, clay pot and bottle brush.

A tray-type incubator consists of a container that is screened or perforated, through which a flow of water permeates to supply the eggs with oxygen and flush away waste products (Figure 2). They are often designed so that water penetrates the tray from below and flows out over the upper edge. Since the eggs lay over a screen, tray-type incubators are ideal for fish eggs that can be injured by movement during incubation. Tray incubators can be stacked and provide easy access for removal of dead embryos. The newly-hatched larvae can drop through the screen holes minimizing handling and removal of the egg shells.

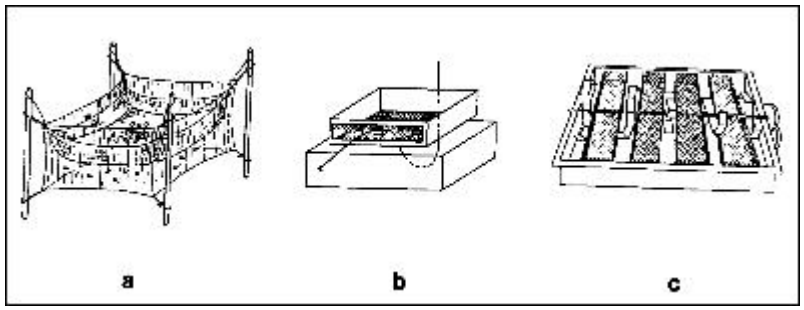


Figure 2. Incubators: a) traditional "happa", framework with fine mesh, b) basket-type, and c) multiple baskets with paddlewheels.

Tray-type incubators were originally designed to hatch trout and salmon eggs. The eggs of salmonid fish are large, non-adhesive and laid in a gravel bed during natural spawning. The eggs must remain still and in the dark since abrupt movements and direct sunlight affect embryonic development. Tray-type incubators also are formed into baskets and commonly used to incubate and hatch channel catfish eggs. The baskets are placed in a water trough, and paddlewheels, which are attached to the trough, provide aeration and gentle circulation of the water. Baskets can also be placed outside the spawning tank and then used as incubators. The "hapa" or net enclosures traditionally used for spawning, egg incubation, and larval rearing of common carp function sirnilarly to basket or tray incubators.

Fish eggs that are non-adhesive and require constant movement are commonly incubated in conical shaped tanks or jars where water flows into the bottom or top of the container (Figure 3). In this type of incubator the eggs are gently suspended and constantly tumble in the lower portion of the jar. The flowing water not only insures that good quality, well oxygenated water is constantly being replaced in the jar, but the tumbling of the eggs keeps them from collecting debris which can lead to fungal infections. These types of incubators can be set in series above a rearing tank. The larvae pour out of the incubators into the rearing tank as they hatch. A soft meshed material can be shaped into a cone and used as an incubator. It is advantageous to use screen because greater surface area is provided for water to flow out, preventing the eggs, yolk-sac larvae or the larvae from becoming crushed. Incubators made of net material require structural support and must be suspended inside a larger tank or placed into the rearing tank.

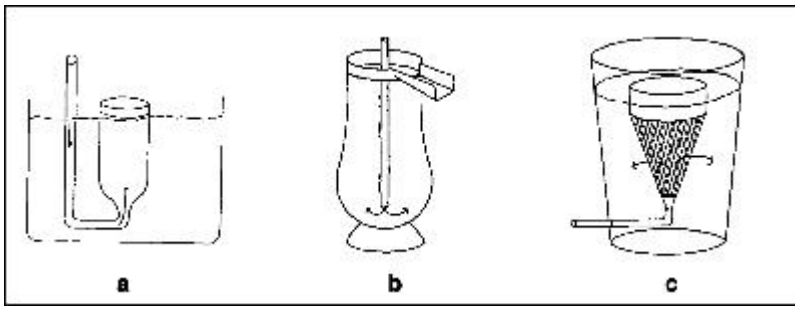


Figure 3. Incubators for non-adhesive eggs:
All three devices provide gentle water circulation.