Reproduction and Genetic Selection

Controlled breeding will obviously be possible only if there is adequate knowledge of the factors governing reproduction of the animal and its breeding behaviour. Lack of such knowledge has hampered the progress of aquaculture of several important species. The extensive culture of Chinese carps, Indian carps, mullets, milkfish, sea-bass, sea-bream, penaeid shrimps, oysters and mussels has been based until recently on 'seed' obtained from natural breeding. Despite advances made in techniques of controlled or semi-controlled breeding, the techniques have not been sufficiently perfected or adapted for large-scale production of seed, with the result that the aquaculturist has still to depend partially or entirely on natural seed resources. There are also species like the eels for which no propagation technique has so far been developed, even though some progress has been made in maturing and spawning under laboratory conditions.

Among the aquaculture species, finfish as a group has received greater research attention in controlled reproduction. The reproductive cycles of almost all fish are regulated by environmental stimuli. Appropriate sensory receptors convey the environmental stimuli to the brain in the form of neural inputs. This neural information, on reaching the hypothalamus, causes the release of hypothalamic peptides known as releasing hormones, which in turn stimulate the pituitary gland to release the gonadotropic hormone(s), which act on the gonads. The gonads in turn produce the sex steroid hormones which are responsible for the formation of gametes, as well as for the regulation of secondary sexual characteristics, nuptial coloration and breeding behaviour.This pattern of reproductive mechanism provides the basis for methods of induced reproduction, namely the provision of appropriate environmental stimuli and the administration of hormones for maturation and release of gametes.

Reproductive cycles

The large majority of aquaculture species are seasonal breeders, although some breed intermittently or continuously. Seasonal breeding is generally related to climatic seasons. For example, most fresh-water fish of temperate zones spawn in spring and early summer, but the salmonids spawn in winter. Obviously the fishes integrate their own reproductive functions with environmental cycles.The breeding season appears to coincide with environmental conditions that are most conducive to the survival of the offspring.These favourable factors, that act as cues for a suitable breeding season, affect the central nervous system and through it the pituitary and the gonads. Photoperiod, temperature and rainfall are important factors involved in regulation of the reproductive cycles.

Mechanisms of reproductive timing vary very considerably among species. For example, in salmonids that spawn in the autumn, gradually increasing photoperiods followed by short photoperiods or decreasing photoperiods have a major role in regulating the cycle. Temperature has an important role in the reproductive cycle of cyprinid species. Gonadal recrudescence takes place in Indian carps during the period of the year when both photoperiod and temperature are increasing. Changes in the volume and velocity of water, flooding of shallow areas and dilution or replacement of water are also considered to be important factors.Warm temperatures and long photoperiods appear to affect also the reproductive cycle of Chinese carps. A review of available information would appear to show that in the majority of cases gonadal recrudescence is regulated chiefly by seasonal variations in photoperiod and temperature, while spawning may be controlled by temperature and/or rainfall.

The age of sexual maturity varies widely between species. For example, tilapia species become mature within a few months, whereas others may take a few years.The same fish may mature earlier in a warm climate and much later in colder climates; examples of this are the common carp and the Chinese carps. The common carp, which takes three to four years to mature in Europe, takes only a year to attain maturity in tropical regions. Chinese carps that take five to seven years to mature in Europe become mature in one to three years in tropical and subtropical conditions.

Some species have only one spawning season, during which they may spawn several times. Others may have two or more spawning seasons. Some species of finfish exhibit well developed parental care, which may consist of incubating fertilized eggs in the buccal cavity of the parent, or guarding the eggs and larvae during development. Many of the species that exhibit parental care lay eggs in nests made of plant or other available material or in hollows dug out on the bottom. Some of the species like the Chinese and Indian carps that are essentially riverine spawners would not spawn in the confined waters of fish ponds or other enclosures. Their gonads develop only up to a certain stage and then remain dormant until resorption sets in. They have however, been observed to spawn in special types of ponds (called bundhs in India) that have a flow of fresh rainwater, inundating shallow marginal areas where the conditions are favourable for the fish to breed. The simulation of conditions in natural spawning grounds may serve to induce certain fish to breed in confined areas. The provision of nestbuilding material for nest-breeding species and the provision of artificial substrates for the attachment of eggs required for certain species are also believed to induce spawning.

Control of reproduction

In aquaculture, the main purpose of controlled reproduction is to achieve sexual maturation and spawning at the time of the year which is normal to that species. As mentioned earlier, some species will not breed in the confined waters of an aquaculture facility. In other cases, maturation and spawning are unpredictable, because of the culture conditions or environmental factors. Controlled reproduction can also be of considerable importance in advancing or retarding the spawning period as required. This can help in making available young ones at appropriate times or of appropriate sizes. A higher level of reproduction control would involve development of the capability to mature and spawn a species at any time of the year, in order to enable continuous production and marketing throughout the year.

The two major types of control that are possible, consist of (i) manipulation of the reproductive cycle and (ii) induction of gonadal gamete release (ovulation and spermiation).

The reproductive cycle is manipulated so as to have gametes available when needed.This may be initiated in the juvenile stage, or advanced or retarded in the adult stage. Altered gonadal gamete release can be achieved by hormonal supplementation, manipulation of environmental factors or the use of special selected strains.

In oviparous animals, embryos are dependent on the egg yolk for their nutritional require ments. Vitellogenesis, or the process of yolk deposition in oocytes, is a seasonal or cyclic phenomenon. All stages of it, starting with the mobilization of lipid from storage sites, the synthesis in the liver of a female-specific glycolipophosphoprotein, vitellogenin, and its eventual deposition in oocytes are known to be gonadotropin-dependent.

The interaction between the brain, pituitary gland, testis and ovary largely mediates the influence of environmental factors on the reproductive development of finfish.The thyroid and interrenal may also have a less important role. The substance formed by the nucleus lateralis tuberis in the hypothalamus, which is responsible for such influence, is the gonadotropinreleasing factor or releasing hormone. In the case of mammalian luteinizing hormone (LH) and follicle stimulating hormone (FSH), the releasing activities for these two hormones have been shown to be present in the same peptide, which consists of a chain of 10 amino acids (Schally and Kastin, 1972). The molecule is referred to as LH-RH.The presence of LH-RH has been demonstrated in certain species of fish (Crim et al., 1978) and it has also been demonstrated that in large doses mammalian LH-RH or its analogues brings about the release of gonadotropin.

Even though attempts have been made with salmonids, the induction of a completely new reproductive cycle has not yet been successful. Chronic administration of gonadotropic hormones can, however, initiate a normal reproductive cycle and assure its progress. By pellet implantation of hormones, it has been possible to advance normal spawning by one year in pink salmon. The release of gametes can be advanced by a single dose of hormone. Similarly, it has been demonstrated that hormone injections can induce late ovulations, as in brown trout, when maturity is blocked by adverse environmental conditions.

As mentioned earlier, the two major environmental factors that affect maturation and spawning are the photoperiodic regime and temperature. Although any definitive conclusions regarding the independent influence of photoperiodism have not been possible, there is enough evidence of the combined effect of these in several species.When, by manipulation of these factors, early maturation is achieved, egg-laying can more easily be synchronized by hormonal injection. This helps in predicting ovulation more precisely and in avoiding ageing of ova, which may occur at high summer temperatures. There is considerable experimental evidence of the independent role of temperature in maturation and spawning. It is believed that spawning is timed to ensure that gametes are released into water whose temperature is within the appropriate stenothermal conditions for embryonic development. While the precise mechanisms by which temperature regulates reproductive development are not known, it is presumed that it acts as a triggering mechanism at the hypothalamic level or alternatively exerts a generalized stimulatory effect on metabolic rate. The influence of rainfall on the spawning of certain species, as referred to earlier, is also ascribed to the combined effect of temperature and photoperiod, plus the dilution of inhibitory elements in the water.

Another means of reproductive control, oriented to spreading egg production over the year, is through the use of selected strains for early or late spawning. Strains have been developed that spawn for much longer periods than normal for the species.There is also the possibility of using in a farm several strains,reproducing at different times of the year, in order to ensure the availability of young throughout the year.

Induced reproduction

As explained in the previous section, the hypothalamus regulates the reproductive functions of the pituitary gland. The correct combination of environmental factors required for maturation, ovulation and spawning, brings about an accelerated release of gonadotropin from the pituitary into the bloodstream. Reserachers have isolated two gonadotropic hormones: one with a low carbohydrate content that induces vitellogenesis and the other, rich in carbohydrates, inducing maturation and ovulation. The surge of gonadotropins that occurs brings about maturational changes culminating in the act of spawning. Environmental conditions required for the initiation of oocyte maturation, ovulation and spawning are much more complex than those for gametogenesis. Very often under culture conditions, the required environmental conditions may not be available, or may not persist for a sufficient length of time for spontaneous maturation to occur. This has led to the development of induced reproduction or hypophysation techniques By the injection of pituitary homogenates (fig. 8.1), the natural gonadotropin surge is simulated, by-passing to some extent the environmental variables of temperature, rainfall, photoperiod, etc. Besides the advantage of regulating the time of spawning, this enables the adoption of other methods of artificial propagation, including hand-stripping, fertilization, incubation, hatching and larval rearing.While hypophysation has been demonstrated to be effective in a large variety of fish species, its major contribution in respect of aquaculture technologies, since its first field application in Brazil in 1935, has been in the inducement of spawning in fishes that do not ordinarily breed under conditions of confinement or do so only under specific environmental conditions. It has now become a common practice in many countries and is utilized widely in the reproduction of finfish, despite the fact that the relevant mechanisms are not fully understood and little standardization of the techniques has been achieved.

Hypophysation

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The mammalian gonadotropic hormones,LH and human chorionic gonadotropin (HCG), are effective in inducing maturation and ovulation in fishes. Although a number of species have been induced to breed by the administration of HCG or a combination of HCG and mammalian pituitary extract, there are certain refractory breeders, like the Indian and Chinese carps, where fish pituitary homogenates or extracts are needed to induce spawning. There are reports of successful breeding of even these species, by using HCG under certain circumstances. The Chinese carps, which have been bred two or three times by administration of fish pituitary extract, will respond positively to injections of HCG. The identification of sex is another important requirement for successful induced breeding. Many species do not have distinctive and permanent sex characteristics.When there are no secondary sex characteristics, detailed morphometric characteristics will have to be used to separate sexes, particularly in the prepuberty stages. After sexual differentiation, it may be possible to distinguish the sexes through examination of the gonads. This will involve the use of endoscopy or biopsy, which is difficult to use on a large scale. Siphoning of eggs and their examination under a microscope, to determine the stage of maturity of females, have been Other possible methods, such as the use of serum markers or detection of circulating vitellogenin, are also difficult to use in the field. Aquaculturists have therefore to depend largely on practical experience and field observations to distinguish the sexes and determine the stage of maturity of breeders. Brood female fish ready for spawning are identified by the well-rounded and soft abdomen and swollen genital opening. The male releases a few drops of thick milt when its abdomen is slightly pressed. As indicated earlier, several species of fish respond to injections of HCG and other mammalian hormones, and these hormones are commercially available to aquaculturists. Many species, which are more difficult to spawn under confinement, need injections of fish pituitary for maturation and spawning. There are differences of opinion regarding the speciesspecificity of the pituitary, but aquaculturists generally prefer to use the glands of the same or closely related species. It is recommended that pituitaries from phylogenetically close donors should be used, when there is a choice. However, common carp is considered a universal donor and its pituitary is being used very widely for both experimental and commercial breeding purposes for several species. Salmon pituitary is also used for breeding a number of species. Though commercially available on a limited scale, a large majority of aquaculturists have to depend on local arrangements for the collection and preservation of the glands.

Glands of the recipient species or of other proven donor species are used. Fractionation and purification of teleost gonadotropins are still in experimental stages. Though potent gonadotropic preparations have been made from fish pituitaries by means of chemical/ ethanol fractionation, they have not been used widely in spawning refractory fish.

Glands extracted from catches of the selected mature donor species are preserved in alcohol or acetone or frozen for storage. Freshly collected glands are first desiccated in absolute ethyl alcohol (changing the preservative several times) and then stored in fresh alcohol at room temperature or under refrigeration. The glands remain active for a period of about two years. Instead of alcohol, the glands can be desiccated in acetone, changing it several times as for alcohol. The desiccated glands are dried in vacuum and stored in that condition or sealed in vials and stored in frozen condition. Acetone-dried glands retain their activity for 6–10 years.The glands can also be preserved by quick freezing, but the most common method of preservation is acetone drying.

Though a number of methods of preparing pituitary homogenates and extracts have been tried, the most commonly accepted method is extraction with distilled water or saline sollution. The glands are macerated in a small volume of water or saline solution and brought up to the desired volume. Distilled water, common salt solution (0.3–1 per cent) and physiological saline can be used, as they all seem to give equivalent results. The homogenate can be used as such for injection, or filtered or centrifuged to obtain filtrate or supernate which can be injected. Extraction with trichloracetic acid (TCA) at low concentrations of 1.25–2.5 per cent for short timeperiods of three to six hours, is reported to provide more complete extraction and better results. But this practice has not received wide acceptance, probably because of the specific requirements of concentration and extraction time. It is reported that higher concentrations and/or longer extraction, can result in denaturation of glycoproteins.

As pituitary extracts are subject to rapid enzymic deterioration, they have to be prepared fresh every time fish are to be bred. This is obviously inconvenient. Methods of preserving extracts have been tried with some success.

One method involves the extraction of pituitary glands in a small volume of distilled water, and refrigerating it for 24–48 hours, after which glycerine is added to make a 2 : 1 ratio with water. The suspension is again refrigerated for

24–48 hours, centrifuged and the supernate stored under refrigeration in airtight vials. Another method consists of grinding acetonedried pituitaries, sieving them through 40–60 mesh/mm^2 sieves and storing in sealed vials at 5°C. Both these techniques are aimed at achieving homogeneous preparations of uniform potency.

Despite its wide use, the dosage frequency and latency period of pituitary administration remains more or less at a trial-and-error stage, and sometimes leads to poor results. This is mainly on account of the variations in the gonadotropic content of the pituitary material used and the stage of sexual maturity of the brood fish, besides the environmental conditions and the stress to which the breeders are subjected. The mode of injection (intraperitoneal or intramuscular) also appear to affect results. Development of an acceptable method of assessing gonadotropic content should greatly assist in determining practical dosages. Though several biological units have been proposed, none seems to have gained wide acceptance.

Gametes and fertilization

Injection of pituitary homogenate or extract is usually given into the dorsal muscles above the lateral line and below the anterior part of the dorsal fin, or the dorsal part of the caudal peduncle. Injections into the body cavity are considered less efficient. The required quantity of the gland is generally administered in two to four doses (one or more preparatory injections followed by one or more final doses). As indicated earlier, suitable environmental conditions, besides pituitary injection, will be needed for ovulation to take place. Temperature, high dissolved oxygen levels and lack of stress are some of the important requirements. The process of ovulation takes some time, depending on the species and environmental conditions. Maturation of the ovum starts when its nucleus starts to migrate from the centre toward the micropyle and undergoes hydration by absorbing fluids. Ovulation starts with the disappearance of the nuclear membrane and ends with the first meiotic division. At the same time, the follicle which attaches the eggs to the wall of the ovary splits and releases the eggs into the cavity of the ovary, from where it can freely flow through the genital opening.

Many of the fish that are treated with gonadotropic hormones start to spawn in the presence of active males after normal ovulation. The eggs are fertilized by the male breeders and the fertilized eggs can be collected easily for hatching.Where such induced spawning does not occur, it becomes necessary to strip the sex products from the females and males and artificially fertilize them. Ripe ova remaining unspawned for long periods after ovulation become over-ripe and do not develop normally.

It is also not uncommon for the phenomenon of 'plugging' to occur in gravid females subjected to overdoses of hormone. In such cases, natural spawning cannot be accomplished, since a mass of ovarian eggs forms a plug at the urinogenital opening, preventing the free flow of eggs. Stripping will be necessary to obtain eggs from such fish. Stripping and artificial fertilization are necessary also for fish with sticky eggs like the common carp. Such eggs will have to be treated to dissolve the sticky layer, so that they can be incubated in suitable incubators.

A quick washing with a weak tannin solution after the eggs have swollen will be effective in removing the stickiness of the eggs. Common salt and carbamide (urea) solution can also be used for removing the sticky layer.

The ovulated egg which has undergone the first meiotic division will have the second meiotic division when the sperm penetrates it, ending in the extrusion of the second polar body. Further embryonic development leading to the formation of the first somatic cell completes the process of fertilization. The time available for the ripe egg to become fertilized is rather limited in most fresh-water fish, as the eggs swell rapidly in water and this results in the closure of the micropyle.The time available for common and Chinese carps is about 45–60 seconds. In saline solution the eggs seem to remain fertilizable for longer periods, up to several minutes.

The sperm, which is immotile in the testis, becomes motile on contact with the medium in which fertilization takes place. The duration of the activity of spermatozoa varies with the species, but is generally not longer than a couple of minutes. In the males of most species, dense semen with highly motile spermatozoa can be obtained without hormone injection. Administration of pituitary extracts brings about thinning of the seminal plasma and would facilitate spermiation. Relatively large numbers of spermatozoa are needed to fertilize an egg. For example, the requirement of a trout egg is reported to be 10 000–300 000 spermatozoa and of a carp egg 13000–30 000. This is due to the fact that the spermatozoon can penetrate at only one place, i.e. the micropyle, and the dis-

tance that can be covered by a trout spermatozoon during its life span (2 mm) is often less than the circumference of the ovum which is about 15–20mm. the probability of its reaching the micropyle is therefore low, if the motility is less. The number of spermatozoa compensates for the low motility. It is necessary to take special care in regulating the quantity of water added to the sexual products during fertilization. If too much water is added, many of the sperms will not be able to reach the micropyle. On the other hand, if sufficient water is not added, the micropyle of an egg may get blocked by other eggs, due to crowding, preventing the sperm from entering it.

Preservation of gametes

In many species, the maturation of gonads in the two sexes is not synchronous. Males often show testicular recrudescence earlier during the season. Because of this, ripe males occur during the beginning of the season, when the females are not yet mature and ready for spawning. The reverse situation occurs during the end of the breeding season. Under such circumstances, it will be most advantageous to have a suitable means of preserving the gametes for artificial fertilization, when needed. Methods of gamete preservation would also help in the initiation of genetic selection programmes, by providing easy access to a reserve of genetic material of known and desired qualities.

Cryopreservation with liquid nitrogen, used widely in the preservation of cattle and livestock sperm, has been tried for the preservation of a number of species of fish. Blaxter (1955) reported successful fertilization of fresh eggs with cryopreserved (-79°C) sperm of Clupea harengus. Sections of ripe testis were stored in 80 per cent sea water containing 12.5 per cent glycerol as a protector, and the mixture frozen quickly or slowly at 1°C/min to -30°C, then quickly to -79°C (using dry ice). Besides the sperm of rainbow trout, spermatozoa of the common carp, Chinese and Indian carps and grey mullet are among the cultivated species which have been subjected to cryopreservation, which consists of cooling and storing at subzero temperatures of liquid nitrogen (-196°C), using dimethyl sulphoxide, glycerine, ethyl glycol or other cryoprotectants and diluents (Harvey and Hoar, 1979). Attempts at cryopreservation of ova have not been as successful as for sperm. Zell (1978) reported the first successful cryopreservation of unfertilized ova and zygotes of salmonid fish. Ova frozen in liquid nitrogen at -20°C for five minutes proved to be fertile, and zygotes frozen at -50°C survived the exposure. All subsequent attempts have failed.While it is difficult to predict possible advances in cryopreservation of fish gametes, it would appear that the results so far indicate only the feasibility of short-term preservation of semen or the prolongation of embryonation.

8.5 Use of sex steroids for sex reversal

In certain situations and species, it will be advantageous to restrict fertility.A well-known example is the cichlid tilapia, which attains maturity at an early age and breeds repeatedly at short intervals, overpopulating ponds and other rearing facilities. This results in stunted populations, as energy is expended for reproduction rather than growth. Among the techniques that can be employed for restricting fertility is the application of hormones to produce monosex populations. Androgenic and oestrogenic steroids are used for masculinization of genotypic females and feminization of genotypic males (Jalabert et al., 1974; Guerrero, 1975, 1979; Shelton et al., 1978). Genotypic female fry of the species of Sarotherodon (= Tilapia), when fed on methyltestosterone and ethinyltestosterone have become males. Similarly, monosex female tilapia have been produced by treatment with oestrone, ethinyloestradiol and stilboesterol. While the feasibility of sex reversal by steroid administration has been demonstrated, the percentage of fish that underwent sex change in any treated group varied greatly. Since the presence of even a small percentage of the opposite sex in a population is sufficient to initiate uncontrolled breeding, the value of the results achieved so far becomes less significant. Similar experiments to produce monosex fish have been conducted with salmonids and other species.

Sex inversion of the protogynous species of Epinephelus (E. tauvina) has been accelerated to produce male brood stock from three-year old females, by oral administration of methyl testosterone. Production of all-female eggs is now a common practice in a number of rainbow trout hatcheries (see Section 17.1.2). The initial functional males required for fertilizing ova from normal female brood stock are obtained by sex reversal, by treating with 17 methyltestosterone through immersion or incorporation in starter feed in the fry stage.