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with a foreword by

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Abstract — The book summarizes current knowledge of the reproductive endocrinology of fish, and applies this knowledge to a practical end: manipulation of reproduction in captive fish that are grown for food. The book is written in a deliberately "nonacademic" style and covers a wide range of topics including breeding, manipulation of the environment to induce breeding, conservation of fish genetic resources, and techniques used for marking and tagging broodstock.

Résumé — L'ouvrage fait le point des connaissances actuelles en matière d'endocrinologie de la reproduction du poisson dans le souci d'une application bien concrète : la manipulation de cette fonction pour les besoins de la pisciculture. Le livre est rédigé dans un style délibérément accessible au non-spécialiste et couvre un large éventail de sujets, y compris l'alevinage, la manipulation du milieu pour créer les conditions favorables à l'élevage, la conservation des ressources génétiques piscicoles et les techniques de marquage et d'étiquetage des stocks reproducteurs.

Resumen — El libro resumen el conocimiento actual de la endocrinología reproductiva del pescado, y aplica este conocimiento a fines prácticos: manipulación de la reproducción de pescado cautivo para fines alimentarios. El libro está escrito de forma deliberadamente no académica y abarca una amplia variedad de temas, entre los que se cuentan reproducción, manipulación del entorno ambiental para inducir la reproducción, conservación de recursos piscícolas genéticos, y técnicas empleadas para marcado y etiquetado de poblaciones de reproducción.

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Foreword

It is now 15 years since the International Development Research Centre (IDRC) published *The Theory and Practice of Induced Breeding in Fish.* During those years, we have witnessed the rapid development of both scientific knowledge and practical techniques for controlling reproduction in economically important fishes. Much of the progress in the last decade, particularly in reproduction of tropical species, has resulted from support for research and development activities by agencies such as IDRC, the Canadian International Development Agency (CIDA), and the United States Agency for International Development (USAID). Furthermore, this progress could not have been achieved without the cooperation and interest of aquaculture research institutions and agencies in countries where tropical aquaculture is practiced, including Brazil, People's Republic of China, India, Malaysia, the Philippines, and Thailand.

Seed production is one of three key biological areas that must be addressed in the development of a viable aquaculture system — the others are diet and fish health. Seed production embraces several specific areas, including *broodstock husbandry, genetic selection, induced breeding, gamete storage, fertilization techniques, controlled sex differentiation, incubation,* and *larval rearing to stocking size.* This manual focuses on endocrine and environmental techniques for inducing spawning in tropical fish, but also provides information on assessing broodstock maturity, tagging broodstock, and sperm preservation. Planned *inhibition* of reproduction is necessary in the culture

of some fish such as tilapia. This is a separate topic not covered in this book but it has recently been reviewed by Zohar (1989)[1].

Since the first use of homogenized pituitary glands to induce spawning in indigenous Brazilian species in the 1930s, induced-spawning technologies have evolved through several generations. Methods have been developed for preserving fish pituitary glands, for preparing gonadotropic extracts, and for purifying human chorionic gonadotropin. Subsequent research explored the feasibility of using steroid hormones that act directly at the gonadal level, as well as the use of antiestrogens as estrogen-feedback inhibitors. This latter topic shows enough promise to warrant further research to explore the endocrine response to several antiestrogens developed for use in human medicine.

In the early 1970s, the characterization and synthesis of mammalian gonadotropin releasing hormone (GnRH) and its analogues led to its successful use in some species and failure or limited success in others. The last decade has also seen the characterization of *teleost*-GnRH and the development of potent analogues based on its structural backbone. Recent research has also shown that, in several groups of teleosts, a dopamine antagonist must be administered along with GnRH to block the inhibitory effects of dopamine and achieve successful ovulation.

Other recent developments that will allow refinement of induced spawning include *new anaesthetic procedures* and *methods for cryopreserving sperm*. New techniques for anesthesia are needed to further minimize the

[1]Zohar, Y. 1989. Endocrinology and fish farming: aspects in reproduction, growth and smoltification. Fish Physiology and Biochemistry 7, 395–405.

endocrine stress response to handling, and may use recently introduced anesthetic drugs such as metomidate hydrochloride. Cryopreservation of gametes and embryos is of great importance, making it possible store valuable gametes (e.g., monosex sperm), to use gametes in a later spawning season, to transport gametes, to distribute progeny-tested gametes, and ultimately to eliminate male broodstock, as has happened in cattle farming in many counties.

Along with the development of endocrine techniques, interest has recently increased in using *environmental optimization* to bring cultured fish to spawning readiness and, in some cases, to spawning. Culture conditions differ from those in the natural environment and are often not optimal for the final stages of sexual development and the production of high quality gametes. On the other hand, providing optimal conditions for broodstock can eliminate or at least reduce the need for pharmacological intervention. In species such as trout and turbot, where the environmental regulation of sexual development is already well understood, culturists now maintain several individual stocks under environmental regimes that allow sequential spawning throughout the year. Further research on the environmental regulation of reproductive development in tropical species will go a long way to promoting development of technologies for year-round seed supply.

Although much has been achieved, we can expect further developments in induced breeding and associated technologies. Some might argue that it is difficult to transfer "high-technology" solutions to developing countries. My own experience, particularly in Brazil and Thailand, is that these reproductive technologies *can* be introduced into government or academic aquatic facilities and that, once established, the technologies can be

successfully transferred to private-sector farmers with appropriate adaptations for local requirements. The basic operating principle, both in the developed and the developing world, is that the new technology will be rapidly adopted as long as it presents a sufficient advantage (especially an economic one) over existing technologies.

New technologies that will see future application in reproduction of tropical fishes include: *slow-release hormonal implants, oral hormone administration, procedures for cryopreservation of embryos and ova, greater use of sex-reversed broodstock to produce monosex gametes and offspring*, and *the use of induced breeding technologies to provide gametes and zygotes in a synchronized manner for associated biotechnologies such as chromosome-set manipulation, cloning, and the production of transgenic fish*. Implementation of these technologies will improve production and, in so doing, further enhance the role of aquaculture in the provision of high-quality protein for human diet.

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Chapter 1

Introduction

When it comes to reproduction, fishes are the most resourceful of animals. No other vertebrate group varies as much in reproductive timing and behaviour, size of gametes, mechanism of fertilization, and care of the young. In spite of having to contend with such a broad range of reproductive strategies, all fish culturists have in common the knowledge that, like any captive animal, fishes need certain environmental and social cues before they will mature and spawn. Take away enough of these cues and they stop reproducing. They may grow perfectly well, but they will never reproduce. Impairment of reproduction in captivity can be partial or total: in some species for example, the gonads may mature only so far; in others, one sex may mature sexually and even produce viable gametes where the other does not; in many species, there may be few signs of gonadal maturation at all, long after wild fish of the same age have spawned repeatedly.

Fish farmers need fry or "seed" predictably and in very large numbers. When the numbers of wild-caught fry fall short of what farmers need to stock their ponds or cages, and ultimately to make a profit, producers must somehow induce adult fish to reproduce in captivity. The better the reproductive physiology of fishes is

understood, the better are the chances of success with induced reproduction.

The challenge today is to balance the cost of providing a sufficiently "natural" captive environment against the effectiveness of intervening with hormones; as we learn more about the cues that lead to spontaneous maturation and spawning, the trend for many species will be away from hormones and toward environmental manipulation. A complementary approach, where environmental manipulation and more attention to broodstock requirements for diet, water quality, and holding conditions are combined to enhance the outcome of hormone techniques, will also be productive.

During the past decade, our knowledge of social and environmental cues in fish reproduction has advanced steadily; at the same time, methods for hormone-induced reproduction of fish have become more and more refined. There is a fundamental and very important connection between these two developments. As we shall see in detail later, the successful hormone-induced reproduction techniques of today are based on intervention at precisely the point where environmental cues are translated into the first of a series of chemical messages that ultimately result in final maturation and release of ripe eggs and sperm.

We look at this chain of hormonal messages in detail later; we must understand their progression from sense organs to hypothalamus to pituitary to gonad, and even how primary chemical messengers are transformed, released into the environment, and recirculated as external chemical messengers.

First, however, let us illustrate some important general points about environmental cues and the hormonal chain of events that they set in motion by considering a

fish whose controlled breeding affects lives and economies in more than a dozen countries where it is grown for food — the milkfish.

Lessons from breeding experiments with milkfish

In the late 1970s, when the International Development Research Centre (IDRC) commissioned *The Theory and Practice of Induced Breeding in Fish*, the first few milkfish were just beginning to be spawned in captivity, using gonadotropin from salmon and humans; results were tantalizing but hard to repeat. By the mid-1980s, the emphasis had shifted in favour of gonadotropin-releasing hormones that act right at the interface between the brain and the endocrine system, and successful, repeatable spawnings of captive broodstock began to be reported from several countries. At about the same time, however, better broodstock care in more natural conditions began to yield what was unthinkable only 5 years earlier: naturally spawned eggs from captive broodstock untreated in any way.

There are two lessons here, and they are echoed through this book: an "impossible to spawn" fish can do just that, given enough cues from its environment; and, if providing an adequate environment is economically impossible, forced spawning is often best done using a hormone that acts early in the hormonal chain. These are sweeping generalizations; the rest of this book is devoted to the evidence, from theory and practice, that allows us to make them.

Chapter 2

Fish reproductive endocrinology

The nervous and endocrine systems of fish act together to coordinate reproduction. Figure 1 shows a simplified chain of events beginning with reception of stimuli from the environment and ending with the release of ripe gametes.

Neural events start the chain, and later links are hormonal. Stimuli from the environment — primarily daylength (photoperiod), temperature, and cues associated with rainfall — are processed by sensory receptors; the resulting neural signals, upon reaching the hypothalamus in the base of the brain, influence the pituitary gland through chemical messengers known as *releasing hormones*. These releasing hormones, whose practical importance in induced breeding is now firmly established, stimulate the pituitary to release into the general circulation hormones whose target organ is the gonad. These hormones are called *gonadotropins*, and they influence the production of *sex steroids* in the gonad itself. Sex steroids are responsible for gamete maturation and, if the appropriate environmental and social signals are present, ovulation (or spermiation) and spawning follow.

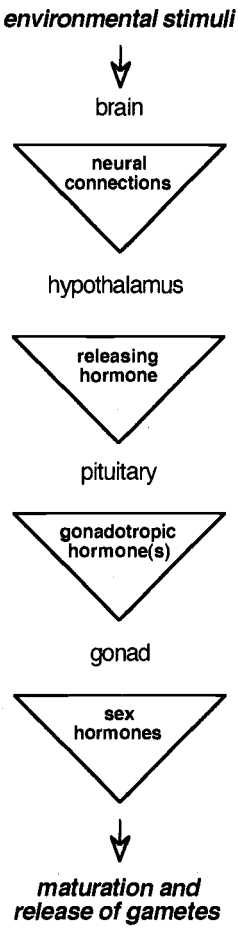

Fig. 1. The hormonal chain of events leading to the release of mature eggs and spermatozoa.

Let us look at these events in enough detail to make today's induced breeding methods understandable. We begin with the production of releasing hormones in the hypothalamus, move from there to events in the pituitary and the release of its gonadotropins, and finally consider gonad maturation and spawning in both sexes.

Anatomy of the pituitary and hypothalamus

The pituitary gland or *hypophysis* of fishes is a discrete lump of tissue attached to the hypothalamus, a region of the base of the brain (Fig. 2). It has two parts: the first or *adenohypophysis* produces a variety of hormones including gonadotropins, somatotropins (growth hormones), corticotropin, prolactin, thyrotropin, and melanotropin; we are concerned only with the gonadotropins. The other part of the pituitary is made up of axons (nerve fibres), whose cell bodies are located in the hypothalamus, and is called the *neurohypophysis*.

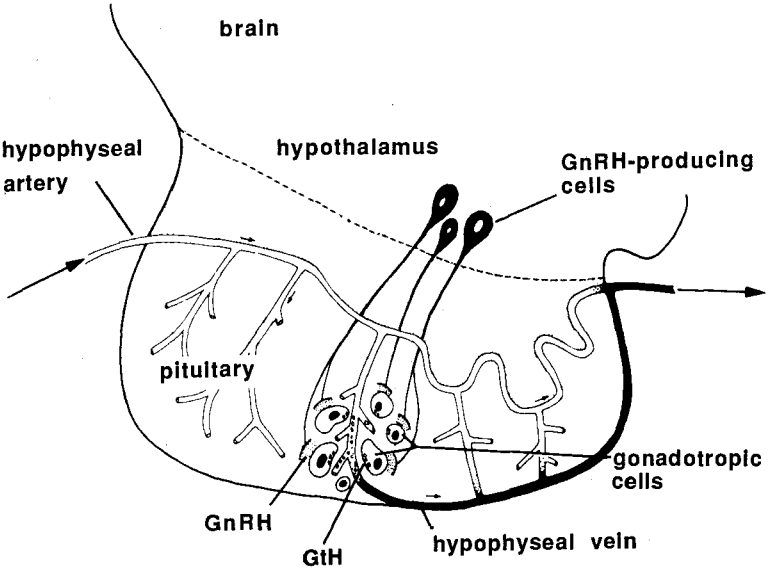

Fig. 2. The teleost pituitary gland and its production of gonadotropin. Blood enters the gland from the hypophyseal artery (left) and exits to the general body circulation by way of the hypophyseal vein (right). Gonadotropin (GtH), produced by gonadotropic cells stimulated by gonadotropin-releasing hormone (GnRH) from the endings of hypothalamic nerve cells, is collected along the way.

It is intimately connected with the adenohypophysis and, for our purposes, is simply a bridge between hypothalamus and adenohypophysis.

Hypothalamic nerve cells, whose axons make up the neurohypophysis, are a specialized type called *neurosecretory cells*. They respond to an electrical signal from the brain by secreting a hormone at the end of the axon: thus an electrical signal becomes a chemical one. The arrangement of these neurons is unique to teleost fishes, because their axons end directly on endocrine cells in the adenohypophysis, and do not secrete their hormones into a portal blood system as in other chordates.

Gonadotropin-releasing hormone

What happens at this junction between the axons of hypothalamic nerve cells and the gonadotropic cells of the adenohypophysis is crucially important to all practical methods of hormone-induced breeding today. A *gonadotropin-releasing hormone* (GnRH) released at the junction acts on gonadotropic cells to promote production of gonadotropins, which in turn are released into the vascular system of the adenohypophysis and then travel, by way of the general body circulation, to the gonad (Fig. 2).

Before looking more closely at GnRH and its actions, we must clarify the terminology. The terms GnRH and LHRH (for *luteinizing-hormone releasing hormone*) are often used interchangeably but, strictly speaking, the term LHRH refers only to a certain kind of GnRH — one that releases luteinizing hormone (LH). The gonadotropins produced by fish are not the same as mammalian luteinizing hormone, although one of them

is, at present, described as "LH-like". GnRH is the generic term, and we should speak of mammalian GnRH, avian GnRH, fish GnRH, etc. When we discuss the use of releasing hormones in fish culture, we need both terms, because many of the hormones are in fact analogues of mammalian LHRH, and some people still use the older term.

Our knowledge of the structure and function of GnRH in fish has increased by leaps and bounds during the past decade. GnRH in fishes, like mammalian GnRH, is a small (10-amino acid) peptide. So far, its structure has been determined for only a few fish — salmon, lamprey, catfish, ratfish, and dogfish — but chromatographic analysis has clearly shown that there are identical or only slightly differing molecules in all fish except the hagfish. (In most species, there are, in fact, two or more forms present, but it is not yet clear if these have different functions. So far, the different forms have been named after the fish or other animal in which they were first identified (Table 1).) Only the mammalian and salmon GnRH are currently used in routine induced reproduction of fish; the salmon GnRH molecule is 80% identical to mammalian LHRH. It is fortunate for fish culturists that GnRH is such a small molecule: as a result, chemical synthesis is economical.

Effect of GnRH on gonadotropin release

What does GnRH do in the fish pituitary? Its main effect is to cause a rise in plasma gonadotropin (GtH), released from pituitary cells whose receptors are stimulated by GnRH. Prolonged exposure of fish gonadotropic cells to GnRH does not cause desensitization and eventual inhibition of GtH release: in practical terms, this means that continuous GnRH administration does not automatically "shut down" the GtH system, as

Table 1. Forms of the gonadotropin-releasing hormone (GnRH) molecule present in some selected fish species.

Species[b]	GnRH form[a]			
	Salmon	Chicken II	Mammalian	Novel
Dogfish	+	+		++*[c]
Milkfish	+	+		
Mullet (striped)	+	+		
Salmon	+*	+		
Sea bass	+			
Ratfish		+		
Sturgeon		+	+	
Catfish (Thai)		+*		+*

Source: Adapted from Sherwood (1990), Lovejoy et al. (1992), and Ngamvongchon et al. (1992).

Note: Identity in most cases is proposed on the basis of elution time with a standardized high-pressure liquid chromatography (HPLC) protocol and relative immunoreactivity with antibodies that recognize the forms differentially.

[a]For those marked with an asterisk (*), the actual structure has been confirmed by peptide sequencing.

[b]See Appendix for scientific names.

[c]Two novel forms have been identified.

happens in mammals. The release of GtH from the pituitary is influenced by at least one other brain hormone, *neuropeptide Y* (NPY), which was first discovered in mammals and recently found in fish. This hormone appears either to stimulate or inhibit GtH release, depending on the reproductive state of the animal: much further work must be done before we will know whether NPY has any practical role in induced breeding.

Inhibition of gonadotropin release

Many biological systems, particularly hormonal ones, have control factors that inhibit as well as those that stimulate (Fig. 3). Although we have seen some evidence for inhibitory action of NPY, and other regulatory mechanisms are still being discovered, the strongest evidence

for gonadotropin inhibition in fish is for *dopamine*. Dopamine, a neurohormone closely related to norepinephrine and epinephrine, is found in high concentrations in nerve endings in certain locations in the hypothalamus.

Like many other brain chemicals (including serotonin, norepinephrine, acetylcholine, and histamine), dopamine's function in vertebrates is still not fully known, but researchers have shown that this simple chemical inhibits release of pituitary gonadotropin in various species of Chinese carps, catfish, and goldfish. They did this by blocking dopamine action with an injection of a dopamine-receptor antagonist (domperidone or pimozide), or by depleting dopamine reserves using the drug reserpine, resulting in an increased and more

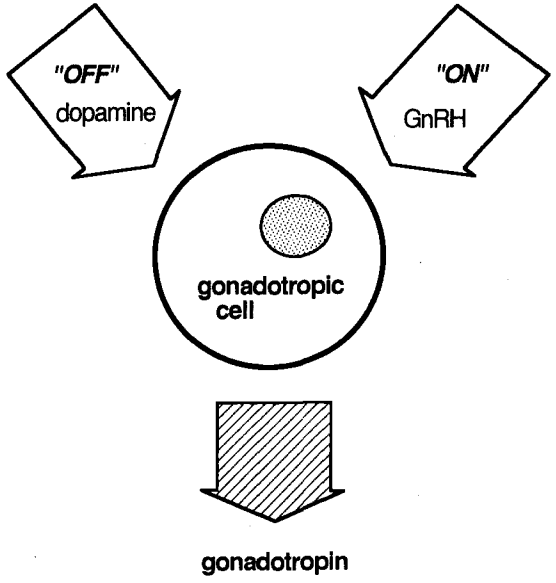

Fig. 3. Stimulation and inhibition of a gonadotropic cell: GnRH stimulates the cell to release gonadotropin; dopamine inhibits it.

reliable release of GtH when the fish were injected with GnRH. As we shall see, this experimental approach provided fish culturists with a highly effective practical tool for induced reproduction.

Fish gonadotropins and their actions

So far we have described today's model for control of gonadotropin release: electrical signals from the brain release chemical messengers that, in turn, have an "on" effect (GnRH) or an "off" effect (dopamine) on the pituitary's release of gonadotropins. What are fish gonadotropins and what do they do?

Gonadotropins are hormones that stimulate growth and development of the gonads. They are glycoproteins (proteins with attached sugar molecules) produced by the pituitary gland. The number and type of gonadotropins in fish have been controversial and the topic of heated discussion. Only recently has real progress been made on this question. We now know the exact structure of two gonadotropins in several fish species, primarily salmonids. They have been named *gonadotropin I* and *II*, and are structurally similar, respectively, to *follicle stimulating* and *luteinizing hormone* in mammals.

The elucidation of the structure of these gonadotropins, along with the development of techniques to measure them individually, is opening the door to some interesting research, and older models of how gonadotropin(s) work in fish are seriously compromised. For example, we do not know if the "carbohydrate-poor" gonadotropin previously considered the "second" gonadotropin is related to one of the now-known gonadotropins, is a third gonadotropin, or is not a

gonadotropin at all. More importantly, radioimmunoassays used in the past to measure gonadotropin appear to detect primarily gonadotropin II, but the degree of crossreactivity with gonadotropin I in many of the assays is not yet known.

So far, we can only measure gonadotropin I reliably in salmon, but, based on this research, Swanson (1991) has started to reconcile the older endocrine models with the new information. We have incorporated this discussion in the models of fish reproduction that we present, but the reader should be aware that knowledge of the field is now changing very quickly.

Maturation of the gonad

Maturation of the fish gonad is a process where tiny incipient sex cells develop, together with accessory tissue, into large organs with mature sperm or eggs that can be spawned. Maturation may occur once in a fish's life or many times a year, depending on species and conditions. Gonadotropins regulate the formation and maturation of gametes indirectly through steroid hormones produced by the gonad itself, with any number of variations in the pattern of events, depending on seasonality, cyclic rematuration, and even location of developing oocytes within the ovary.

Maturation has various stages and different sex steroids are important at each stage, but gonadotropins influence the production of all of them. Depending on the maturity of the fish, the gonad will be more or less responsive to gonadotropin, it will produce different steroids, and its sensitivity to the various steroids will change. The steroids that the two sexes rely on for gonad growth are different, even though it is the same

gonadotropin that triggers their production in both cases.

Gonadotropins have a central role in all fish studied so far. Historically, artificial supplementation has been the most effective and generally applicable means of inducing maturation. More recently, however, analogues of GnRH (GnRHa), which act one step further up the hormonal chain by inducing release of the fish's own gonadotropins, are promising to be even more widely applicable and reliable. Steroids, on the other hand, have not found general use.

Although research on the reproductive physiology of fish has begun to decipher parts of the complex puzzle of maturation in fish, much of our knowledge is based on a few species, and much remains to be learned. The picture we present here may be more or less applicable to a given species; the farmer should certainly expect differences as well as variability between individual fish. The following is a generalized model of maturation in farmed tropical and semitropical fish. However, because so much of our present understanding of fish reproduction is drawn from salmonids, we also include references to this cold-water group to illustrate the tremendous diversity of reproductive strategies.

Gamete maturation and release in females

Gametes develop and mature in the female fish through a series of stages, depicted in Fig. 4, before being released during spawning.

Oogenesis

Oogonia are cells that give rise to *oocytes* in a continuous process called *oogenesis* (Fig. 4, A) and are found in the ovary throughout the life of the animal. To produce

oocytes, oogonia undergo division of genetic material that reduces it by half (two copies are present in the adult, whereas only one copy is present in eggs and sperm). This is the process of *meiosis*. At about the time oocytes enter the early stages of meiosis, they become surrounded by a layer of epithelial cells called the *follicle*, and meiosis stops at this stage as the oocyte enters a long stage of cytoplasmic growth.

In fish that reproduce more than once, oogenesis goes on at various rates throughout the fish's life. The hormonal control of oogenesis is poorly understood and there are no practical endocrine techniques to specifically influence the process.

Primary oocyte growth

The early development of the follicle and its oocyte are independent of pituitary gonadotropin, and regulatory influences during this period are not known. Growth is due mostly to proliferation of cellular components, and does not involve significant input of finished material from outside the oocyte. By the end of the primary growth period (Fig. 4, B), the typical teleost oocyte has increased several hundred-fold in size, to a diameter of 100–200 μm, and is called a previtellogenic oocyte.

During this growth period, the follicle cells differentiate to form a glandular *granulosa*, separated from the oocyte by a *zona pellucida* containing numerous microvilli of the oocyte, and surrounded by an outer *theca*, recruited from surrounding tissues (Fig. 5). These regions all play a role in the further development of the oocyte, either producing or acted on by hormones.

The primary growth process continues throughout the life of fish that are repeat spawners and previtellogenic oocytes are present in the ovary all year. If only

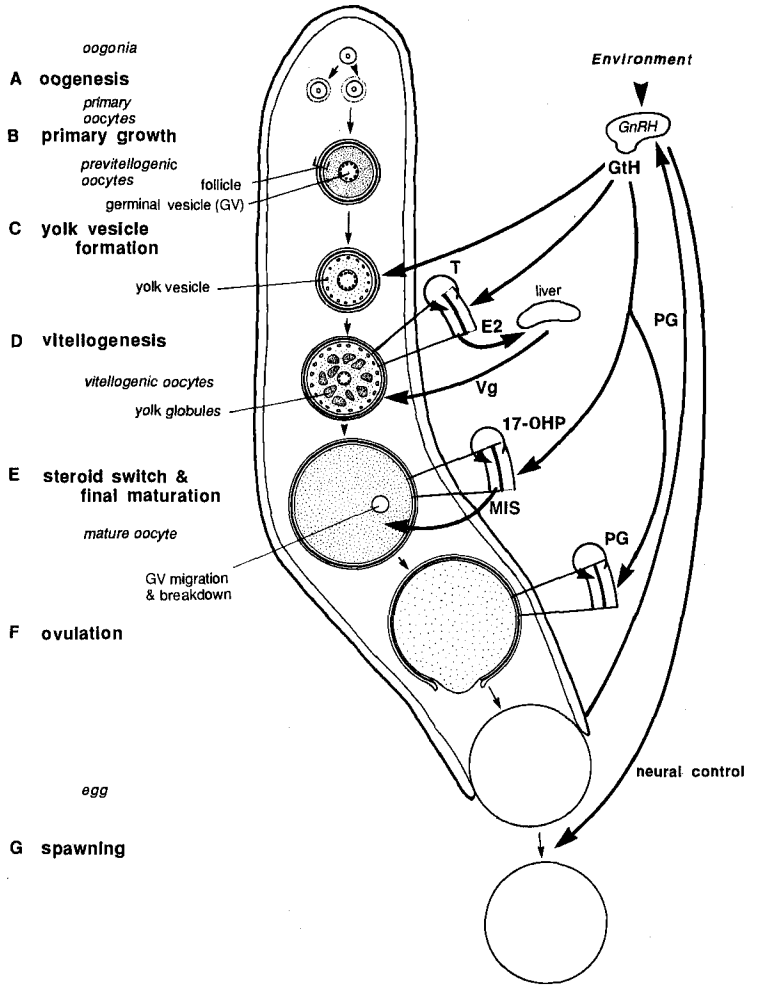

Fig. 4. Maturation of the fish oocyte in the ovary and related hormonal control. Environmental cues act through the brain and gonadotropin-releasing hormone (GnRH) to induce release of gonadotropins (GtH). GtH stimulates the oocyte and then the follicle to produce other steroid hormones: testosterone (T), estradiol (E2), 17 hydroxy-progesterone (17-OHP), and the maturation-inducing steroid (MIS). Vitellogenin (Vg) is produced by the liver in response to E2. Prostaglandin (PG) is produced in the follicle and by the oviduct. (See text and Figure 5 for further details.)

previtellogenic oocytes are present, the ovary is considered immature, although we have to be careful to distinguish between those that are immature because of age and those that are immature because of season. Because the regulation of primary oocyte growth is not understood, this phase of maturation cannot be controlled artificially at present.

Yolk vesicle formation

At some point in the year, in response to environmental cues — which may include changing daylength, temperature, or rainfall — there are periodic surges of gonadotropin in the blood that induce a portion of the previtellogenic oocytes to develop further. The first sign

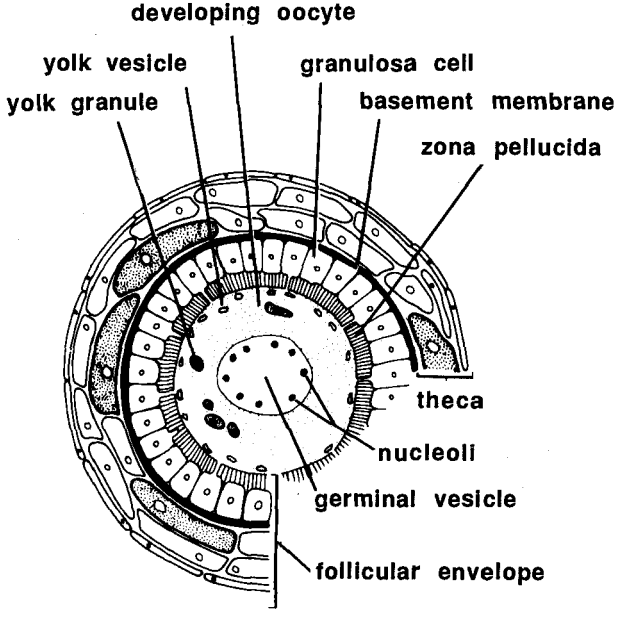

Fig. 5. The developing oocyte in its follicle: steroid synthesis, stimulated by gonadotropins, takes place in cells of the granulosa and theca. (Redrawn from Hoar and Nagahama (1978).)

of this next stage is the appearance of yolk vesicles in the oocyte cytoplasm. These contain glycoproteins formed within the oocyte, and will eventually become cortical alveoli that are expelled into the perivitelline space around the egg after fertilization. This process is sometimes referred to as "endogenous vitellogenesis," but because the material in the yolk vesicles in neither true yolk nor produced from vitellogenin, a better term is simply *"yolk vesicle formation"* (Fig. 4, C). GtH triggers yolk vesicle formation; it is not yet clear which form of GtH is the most important for this function.

Vitellogenesis

Sequestration of the phospholipid, *vitellogenin* (*Vg*), from the bloodstream and the accumulation of *true yolk* in *yolk globules* follows shortly after the start of yolk vesicle formation. The term for this next phase of growth, which represents the major growth of the oocyte, is *vitellogenesis*. This important process involves synthesis of vitellogenin (yolk-protein precursor) in the liver, delivery of vitellogenin to the oocyte via the bloodstream, and uptake and chemical alterations to form yolk protein (Fig. 4, D).

GtH I induces the thecal cells of the follicles to produce testosterone (*T*), which is converted to an estrogen, 17β-estradiol (E_2), in the granulosa cells. E_2 travels to the liver in the blood and stimulates production of Vg, which returns to the ovary, also by the bloodstream. Oocytes sequester Vg as yolk proteins in *yolk globules* and increase in size — the uptake of Vg by the oocytes is facilitated by gonadotropin.

The plasma level of GtH I is high during this phase of maturation, whereas levels of GtH II (also capable of producing the same maturational effects) are low.

Testosterone and estradiol act on the pituitary in "feedback loops" to regulate GtH release, but so far this has only been demonstrated for GtH II.

In some species, vitellogenesis can be triggered and accelerated by environmental manipulation. In trout, manipulation of photoperiod can both trigger and accelerate vitellogenesis. In carp, on the other hand, temperature is more important than daylength, and an increase in temperature can both trigger and accelerate vitellogenesis.

Vitellogenesis can also be artificially accelerated by repeated small injections of GtH or by prolonged-release implantation of GnRH and testosterone. These techniques are not very refined yet and no reliable hormonal techniques are available for triggering vitellogenesis.

Culture conditions strongly influence vitellogenesis. Limited or low-quality food, crowding, and stress can cause *atresia* (resorption of vitellogenic oocytes), resulting in a lower final number of mature oocytes, or they may even prevent triggering of the process in the first place.

Steroid switch and final maturation

Final oocyte maturation in many warm-water species is rapid — it usually takes less than 24 hours — and involves resumption of meiosis, migration of the germinal vesicle (nucleus) to the edge of the oocyte, and *germinal vesicle breakdown* (GVBD), an event that is useful in judging oocyte maturity (Chapter 5). Meiosis then stops again, and the oocyte is now mature, ready for expulsion from the follicle (*ovulation*) and fertilization; it has become an *egg*.

The trigger for the end of vitellogenesis is not very well known, but there is a marked change in hormone

sensitivity and production. GtH II is now secreted in larger quantities than before, while secretion of GtH I is reduced. The follicle produces *maturation-inducing steroid* (*MIS*) instead of estradiol in response to GtH II, and the oocyte starts being receptive to this new steroid. In most fish studied so far, the MIS is a form of progesterone called *17*α,20β-dihydroxy-4-pregnen-3-one (17,20 P) — produced while the enzyme normally responsible for estrogen manufacture is inhibited. How this "steroid switch" is regulated is still a matter for research.

The MIS induces a number of visible changes in the oocyte during final maturation. In addition to germinal vesicle migration and breakdown, in some species, the hormone causes an increase in oocyte diameter due to the uptake of water into the cytoplasm, and may also cause changes in the appearance of the yolk. These gross changes can be very useful in assessing the maturity of the fish — the ovary becomes swollen and soft, for example (see Chapter 5).

Ovulation

Final maturation is followed by *ovulation*: the egg bursts free from its follicle (Fig. 4, F) into the ovarian lumen and is ready to be expelled into the surrounding water for fertilization. In all fishes studied so far, ovulation is controlled by F *prostaglandin* (PG) hormones and/or other related compounds released from the follicle and surrounding tissues on stimulation by the MIS. A surge of GtH II is required for this process.

Once ovulated, the eggs of different species remain fertile within the ovary or body cavity for periods varying from less than an hour to several days. After this

time, the eggs become "overripe" and start to disintegrate. This characteristic must be well understood to optimize induced breeding. In a fish where ovulated eggs remain fertile only briefly (carps are a good example) spawning or stripping must take place quickly. Ideally, hormone injection must be timed to produce ovulation at a convenient time of day.

Ovulation and over-ripening of eggs held in the body after ovulation are highly temperature-dependent. Time to ovulation after the final hormone injection has been documented for different hormones and water temperatures for many fish cultured in the tropics. This information can be used to schedule induced reproduction. If the time of day that the particular fish species spawns naturally is known, induced ovulation should best coincide with this natural cycle.

Final maturation and ovulation are the stages of reproduction most commonly induced in hormone therapy. A variety of protocols are effective, including GnRH and GtH supplementation, which are the most common (see Chapters 3 and 4). Environmental manipulation is also effective for inducing these parts of maturation in a growing number of species (see Chapter 6).

Patterns of Maturation and Induced Reproduction

The reproductive patterns of fish vary greatly. This has implications both for the model of maturation that we have presented and for induced reproduction. We discuss only females here, because this sex is the most affected.

First, fish may spawn once a year or many times. The ovary of a fish that spawns once a year is "group

synchronous" — a group of the available previtellogenic oocytes mature more or less synchronously once a year. Fish that spawn a small number of eggs at short intervals throughout the year have "asynchronous" ovaries — oocytes of all stages of maturity are present simultaneously. Many species cannot be fitted unequivocally into either classification, but most are closest to group synchrony. The group-synchronous maturation pattern lends itself well to the model of maturation that we present: the changing levels of the various hormones in the blood can be correlated with morphological changes in the gonad and induced reproduction can be used at the appropriate time to replace whatever step is not working. Asynchronous ovaries, however, contain oocytes of all stages, presumably responding to different hormones simultaneously, and their follicles are producing contrasting steroids side-by-side. Lin et al. (1991) have proposed a model that addresses this enigma, but clearly these fish need much more research. The modes of induced reproduction most likely to succeed in asynchronous species are prolonged release-hormone therapy and environmental manipulation.

Second, the steps of final maturation and ovulation also follow different patterns in different species. This is best exemplified by two contrasting groups, carp and salmon:

- Carp complete vitellogenesis and then wait as "ripe" fish for appropriate environmental cues such as a change in temperature, presence of spawning substrate, and ripe males. These cues result in an abrupt peak of GtH II associated with final maturation, ovulation, and spawning, all of which can occur within 24 hours.

- Salmon, on the other hand, complete final maturation all the way through ovulation, before waiting for appropriate environmental cues to spawn. Secretion of GtH II increases gradually during late vitellogenesis, continues to rise during final maturation and ovulation, and peaks only after spawning has finished.

The implications for induced reproduction are that, for the carp, we commonly provide a "trigger" that "jump-starts" the processes of final maturation and ovulation, whereas in salmon we are only accelerating this process. The *rate* of gonadotropin release may be most important in carp, whereas in salmon the absolute amount of GtH present is the key factor. We could expect differences between the two species in the effectiveness of dopamine inhibitors and prolonged release GnRH preparations. Also, although both fish only respond to hormone treatments for final maturation at a certain minimum maturity (see Chapter 5), this requirement appears to be much more crucial for the carp than for the salmon.

Most tropical and subtropical cultured species are of the "carp" type (Fig. 6): they complete vitellogenesis relatively easily in captivity but require additional environmental and social cues and triggers to complete final maturation, ovulation, and spawning. If these cues do not appear within a certain period, gonadal atresia sets in.

For some species, the environmental conditions and cues needed for vitellogenesis (and the yolk vesicle stage), final maturation, ovulation, spawning, and atresia are at least partially known, and can be manipulated to induce and control reproduction. For example, some fish that spawn in response to seasonal rains can

be induced to spawn in seasonally flooded ponds with spawning nests, and appropriate culture conditions can induce and accelerate vitellogenesis. Identifying and manipulating these cues will be so important in fish culture in the coming decades that we have devoted a chapter to the subject (Chapter 6).

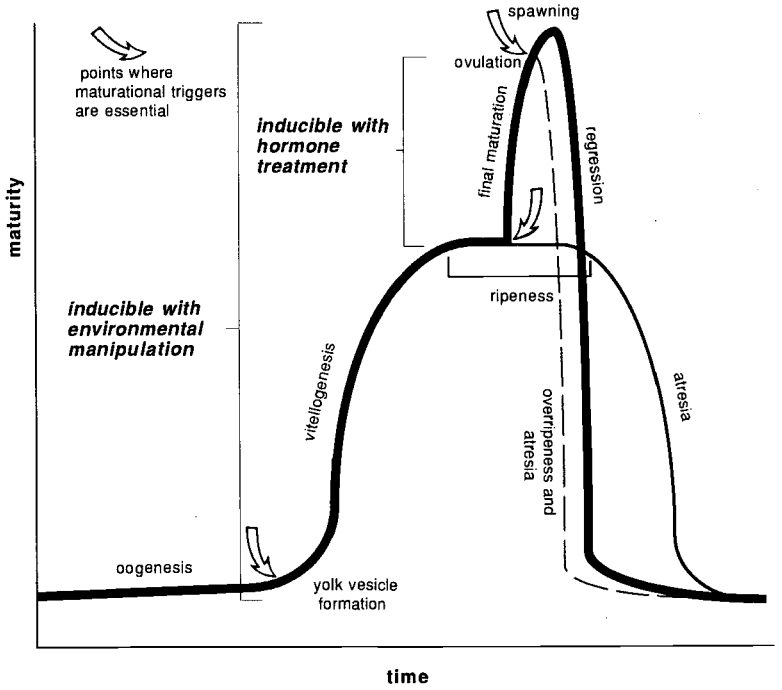

Fig. 6. Female reproductive cycle of "carp-type" fish relative to environmental or hormonal approaches to induced reproduction. Maturation progresses through to spawning subject to the prescence of environmental cues ("triggers") at the start of yolk vesicle formation/vitellogenesis, final maturation, and spawning. The nature and importance of these triggers may differ between species. Final maturation can be triggered at any time during the "ripe" period, but if appropriate cues do not occur, gonadal atresia may set in. In the absence of triggers for spawning or artificial stripping after ovulation, eggs will also spoil, becoming overripe and atretic. Common hormonal techniques for induced reproduction are effective over a smaller range of maturation than are environmental techniques.

At present, however, it is usually easier and more reliable to provide culture conditions that allow the fish to complete vitellogenesis, and then to induce final maturation and ovulation using hormones. The effective range of routine hormone-induced reproduction of fish is thus more limited than the range through which environmental manipulation may be effective (Fig. 6), but the two approaches are not mutually exclusive. The best practice is always to provide the best possible environmental conditions to produce successful reproduction, and then intervene with hormones at the appropriate time if need be.

Spawning

Many species spawn immediately after ovulation as long as the needed environmental and social cues are present (Fig. 4, G). These cues may include the presence and behaviour of spermiating males as well as suitable spawning substrate. In species where manual removal (stripping) of eggs is required, ovulated eggs typically need to be removed within 1 hour or so, before they become overripe and no longer viable. In some fishes, fertility declines drastically within 5 or 10 minutes of ovulation; at the other end of the spectrum, some salmonid species retain ovulated eggs, and stripping can be delayed for up to 1 week.

If not enough hormone is present in the post-GVBD phase of induced reproduction, or inhibitory factors such as handling stress are too great, ovulation may be incomplete and the female can become *egg-bound*, a condition often incorrectly diagnosed as over-ripeness. There is no reliable treatment for this condition, which is often fatal. Because time to ovulation after hormone application is predictable, however, gonadal biopsy can

help in diagnosing the problem, and an additional hormone injection can sometimes induce ovulation.

Our understanding of the hormonal regulation of reproductive behaviour in fish has progressed greatly in the past few years. For example, we now know that both males and females of some species use hormones and their metabolites as *pheromones*, or external chemical signals, that are released into the water and picked up by the olfactory system of other fish and trigger final maturation or spawning. Hormone production is already synchronized with various reproductive events such as final oocyte maturation and ovulation, so releasing the hormones or their byproducts into the surrounding water and having them double as chemical signals makes biological sense.

We have already mentioned prostaglandins as regulators of ovulation. In some fish, these hormones also act as *postovulatory pheromones* that stimulate male courtship behaviour. Females also release a *preovulatory pheromone* (e.g., 17,20 P), which affects nearby males by stimulating GtH release and milt production. Pheromones released by males and affecting ovulation and spawning have not yet been conclusively identified, but are believed to include metabolites of male sex hormones. The important point is that the endocrine system is not just a control mechanism for *internal* reproductive events, but that it is also crucial in modifying social interactions with nearby fish. Manipulating the endocrine system starts a complex process that may go only as far as producing ripe gametes for artificial fertilization; given adequate environmental and social cues, however, the same process may carry through all the way to spawning in captivity.

Regression and atresia

After spawning is finished, the remaining oocytes (other than previtellogenic ones) are reabsorbed in a process called *gonadal regression*. This also happens eventually in "ripe" fish that do not encounter the right conditions for final maturation and spawning (Fig. 6). Reabsorption of individual oocytes can also happen any time during maturation — this is called *atresia*. Poor culture conditions can cause atresia during vitellogenesis, and the condition can also be produced by handling stress during final maturation. Very little is known about the hormonal control of regression.

Gamete maturation and release in males

The formation of spermatozoa in the testis and the formation of milt for spawning are often glossed over in practical treatments of fish endocrinology in favour of a more detailed look at the complex events of oocyte growth, maturation, and ovulation. The research bias is toward events in the female, and it is true that, in many cultured fish, gonadal development in the male goes ahead on its own in captivity. In nature, males with ripe gonads can be found over a much longer period than can ripe females; spermatozoa are ready for weeks during the breeding season, held in the testis until liberated to the outside water during spawning. In many species, however, spermiation and sperm hydration accelerate in the short period before spawning (comparable to the time of "final maturation" in females). Even though males develop sexually in captivity, milt is often scarce and poorly hydrated when needed (carps and milkfish are notorious examples) and an understanding of sperm and milt formation will be useful in overcoming these practical difficulties.

The testis of fish is sac-like and folded, and is lined with a germinal layer of *spermatogenic cells* (*spermatocytes*) that produce mature *spermatozoa* (*sperm cells*) in

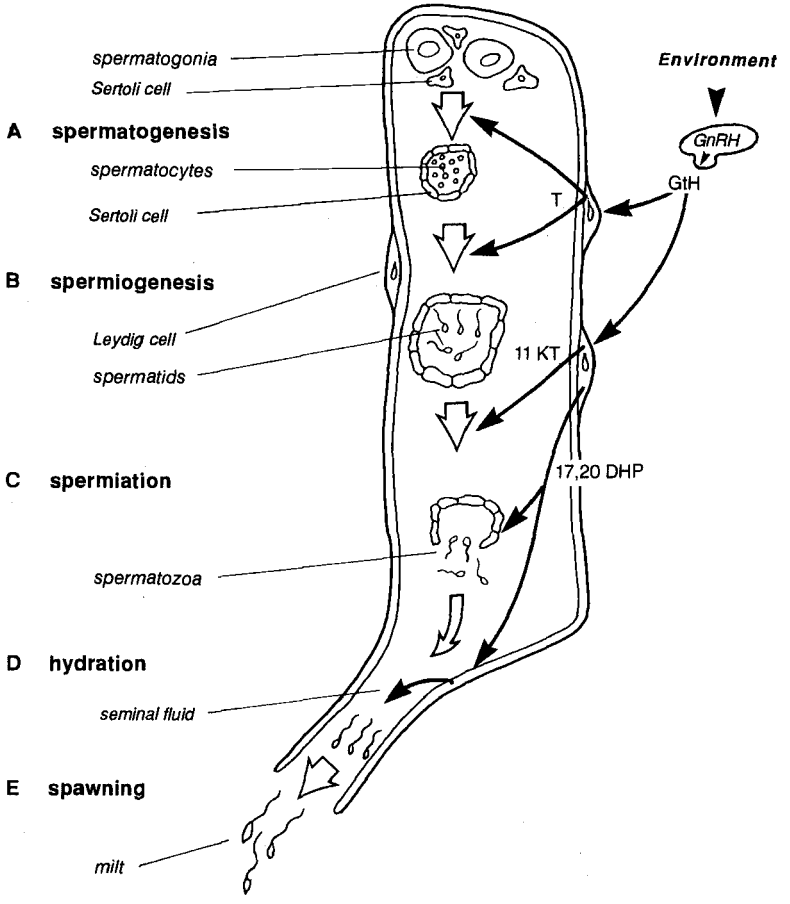

Fig. 7. Testis lobe with progressive stages of maturation showing hormonal control of testis and sperm maturation. Acting through the brain and gonadotropin-releasing hormone (GnRH), environmental cues stimulate the release of gonadotropins (GtH). GtH stimulates Leydig cells to sequentially release the steroid hormones that are responsible for maturation: testosterone (T), 11-keto testosterone (11-KT), and 17,20-dihydroxy progesterone (17,20 DHP). (See text for further details).

a process called *spermatogenesis*. The spermatocytes are enveloped by *sertoli cells* until their liberation as mature spermatozoa within the lumen of the testis in a process called *spermiation*. The last step before release of spermatozoa is dilution (*hydration*) with seminal fluid from the walls of the sperm duct; the resulting sperm suspension is called *milt* (Fig. 7).

GtH induces the *Leydig cells* to produce testosterone (*T*), which causes spermatogonia to divide into spermatocytes within cysts of sertoli cells. Development of spermatocytes into spermatids (spermiogenesis) may continue in the absence of GtH. Mature spermatids are liberated into the testes lumen as spermatozoa. 11-Keto testosterone (*11 KT*), produced by the Leydig cells under GtH stimulation, initiates this process. 17,20-Dihydroxyprogesterone (*17,20 DHP*), also produced by the Leydig cells (and possibly by mature spermatozoa), maintains continuous low-level spermiation, and, just before spawning, induces production of seminal fluid in the sperm duct and accelerates spermiation.

Males are considered "ripe" once spermiation starts, although low level spermiation can go on for several months in some species. Spermiation and sperm hydration increase rapidly when the appropriate environmental cues are present, and are often synchronized with final maturation in females. Males generally ripen readily in culture; if they do not, environmental manipulation or injection of hormones can often induce spermiation. Males need less hormone than females to induce "final maturation," but time to stripping is still important. Once induced, hydration continues until the milt leaks out, and if milt is to be stripped for artificial fertilization, the culturist must have some idea of when volume is highest. In many species, males can be induced to spermiate several times in a season.

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Chapter 3

Practical hormone-induced breeding

Choice of hormone

Fish culturists wanting to induce breeding with hormones will use either a gonadotropin (fish or mammalian, crude or pure), or a GnRH analogue (GnRHa) with or without a dopamine antagonist. The only other avenues being considered at present are using antiestrogens to block inhibition of gonadotropin secretion by estrogen feedback, and manipulating the environment. Research on antiestrogens (clomiphene and tamoxiphen) may have some practical importance in fish farming in the future, but we do not consider it here. Environmental manipulation, on the other hand, is important enough to merit a full chapter (Chapter 6).

The choice of hormone depends on many things, including species, cost and availability, egg incubation and larval-rearing facilities, and training. Often there is a strong historical bias. Broodstock holding also affects the choice of spawning technology. Marine broodstock are more likely to be kept in tanks or cages in clear water, making recapture and identification of individual

fish easier. Facilities for injection or implantation of slow-release formulations are likely to be available. The stringent requirements for rearing marine larvae also make this more likely to be done at a central facility, with fry distributed to growers when they are more robust. By contrast, freshwater broodstock typically have to be captured from turbid ponds, and there is a great advantage to using single injections, because holding space may be limited. Larval rearing is not as difficult and, therefore, less likely to be centralized.

In this chapter, we first discuss using *gonadotropins* from fish and humans, and attempt to balance cost against efficiency. The main emphasis, however, is on the use of *GnRHa*, because this method appears to have the greatest long-term promise for reliable spawning; and, although it may not be universally cost-effective yet, the fact that the method uses easily manufactured chemicals means that wider use will bring costs down as more suppliers enter the market.

First, we must look at some factors that together add up to an enormous source of variability in the results of hormone-induced reproduction. The factors include the *effects of handling stress*, *methods of administering hormones*, and *the method used to mix eggs and sperm*. These are not just afterthoughts that belong in an appendix; any one of them can mean success or failure in controlled reproduction, and too much variability can easily make results unrepeatable.

Importance of stress from handling

Fish try to avoid capture and struggle violently when restrained. The stress of capture and handling has profound effects on blood chemistry and stimulates the

hypothalamus and pituitary, affecting blood levels of gonadotropin, androgens, and the "stress hormone" cortisol. Contact with nets and workers' hands strips away the protective mucous layer and promotes infection. The amount of stress varies tremendously; some species are easily handled whereas others (the milkfish is a good example) leap violently from the water to avoid capture. Handling may result in reduced feeding, infection, or mortality, and there is very strong evidence that the degree of stress in females affects the quality of eggs produced in induced spawning. Even the hidden physiological effects of handling stress are so profound that an induced-spawning procedure using the "right" hormone applied at the "right" time can still fail if broodstock are handled roughly.

One long-term way to increase handling tolerance is *domestication*. Again, milkfish provide an example. In Hawaii, milkfish kept in tanks and handled repeatedly in experiments have become relatively docile. However, this is not a typical production setup. In sea cages, milkfish that have been captive for years but handled infrequently still react violently to netting. In any event, for most fish-culture operations, it is just not practical to spend time training broodstock to accept handling.

Fortunately, even simpler methods will reduce stress and these deserve more attention than they now get. Straight-forward nonchemical methods of reducing stress include the following:

- Avoid overcrowding the fish during capture. A mass of struggling broodstock partially exposed to the air in a seine net is impressive, but is likely to seriously affect reproduction. A solution that has worked for fast-swimming, delicate tuna is to construct barriers and funnels in the holding tank to

allow careful herding of fish into individual capture bags, where they remain during further handling (Kaya et al. 1984). With some trial and error, this idea could be applicable to a variety of holding facilities.

- Never throw fish back into tanks or ponds: they should be carefully placed in the water and then released.
- Moisten hands and all cloth nets or holding slings (*hapas*) before handling fish, to minimize scale and mucous loss.
- Cover the fish's eyes with a wet cloth whenever possible.
- Develop a secure holding technique that minimizes the effect of struggling; often a single handhold at the centre of the fish is much safer than a hand on the tail and one on the head. Fish tend to be more docile when inverted than when upright or on their side.
- Minimize noise during all handling: many fish have a very acute sense of hearing.
- Transport all fish, even freshwater species, in slightly salty water (1–2% NaCl for freshwater species and 80% sea water for marine species) and anesthetize them lightly. Add oxygen if fish density is high or the transport is long.

Using anesthetics to reduce stress

Anesthesia in fishes is usually described as a series of stages, from *light sedation* where fish are easily captured but still responsive to touch, to *surgical anesthesia* where there is no response to stimuli. Fish that are going to be injected for induced reproduction do not

need to be anesthetized, only sedated enough for weighing, biopsy, and injection — usually a matter of a few minutes — and, for this reason, finding the right dose of an anesthetic for a given species must be done by trial and error using published reports as a guide. Excitement profoundly affects ease of sedation, and a dose that works on the first fish removed from an enclosure may have to be increased for fish that have already been stressed as animals are chased and captured.

Descriptions of sedating fish by "placing the fish in an anesthetic bath" omit an essential point: *capture* of the fish from its tank or pen. Yet, for most large broodstock fish used in aquaculture, most of the stress occurs during this first step. This point is often overlooked because many published anesthetic procedures are for small fish, which suffer relatively less from netting; a broodstock female weighing 5 kg, however, is a powerful animal and can injure itself struggling. The real challenge is for culturists to develop ways of exposing these large fish to the anesthetic before they are removed from the tank or cage. For a few freshwater species, it is possible to hand-inject a small volume of concentrated anesthetic solution directly into the mouth of a fish that has been cornered but not yet removed from the tank. Metomidate hydrochloride, a new anesthetic, is very effective this way because it acts so rapidly that sedation occurs in seconds after the solution passes over the gills.

In most anesthetic baths, progressive changes in blood chemistry mean that sedation deepens the longer fish are in the bath; if a fish is left too long, breathing can stop. Fish should be removed to clean water as soon as possible after weighing and injection.

Humans should limit their exposure to anesthetic

drugs: all drugs are potentially hazardous. Little information is available on the effects of long-term exposure to fish anesthetics, and the best practical advice to culturists is to avoid contact with the eyes and mucous membranes, wear long rubber gloves, and limit the number of people touching the powdered or dissolved drug. All anesthetics leave residues in the flesh of fish; broodstock that die during the induced-breeding process should not be eaten.

Anesthetics available for fish-culture work

Only a few anesthetics are available to fish culturists, and all of them are used the same way: dissolved in a small-volume holding bath into which the fish is placed after capture. *Tricaine methane sulfonate* (MS 222®), *2-phenoxyethanol*, and *quinaldine* are all effective on most fishes, although quinaldine is so irritating to fish and humans that it should not be used at all (a less irritating derivative, quinaldine sulfate, has a low margin of safety with many fish and is also not recommended). Chlorobutanol, once widely used, is carcinogenic and should also be avoided. Carbon dioxide bubbled through the water produces sedation and anesthesia in fish and leaves no chemical residues in the tissues, but its action is slow, the margin of safety is narrow, initial excitation is high, and the apparatus required is impractical for sedation of broodstock.

Doses of all anesthetics reported in the literature vary widely with the size of the fish, degree of sedation or anesthesia desired, and water temperature, and should be used as guidelines only. Response to anesthetizing drugs differs widely between species, and these differences are not always just in physiological effect: tilapias, for example, have an unusual (and annoying) ability to shut their mouths in an anesthetic bath,

reducing anesthetic flow over the gills and greatly prolonging time to sedation.

One of the most familiar and widely used anesthetics in fish culture is *tricaine methane sulfonate* (MS 222®; tricaine, TMS). It is available from many suppliers of aquaculture chemicals, and is the only compound registered for use as an anesthetic for food fishes in any country (USA). It is highly soluble in water but is photosensitive and degrades on standing, and is used at 50–100 ppm (mg/L). Tricaine has many drawbacks, and is certainly not the cheapest drug for sedating fish.

Unbuffered fresh water baths of tricaine are acidic and irritate fish, and the long list of physiological effects includes hypoxia and changes in blood electrolytes, hormones, cholesterol, urea, and lactic acid. Some of the irritating effects of tricaine can be eliminated by neutralizing the anesthetic bath using sodium bicarbonate. The buffer should only be added to the working solution; it will precipitate in more concentrated stock solutions and eliminate the anesthetic effect. Sodium bicarbonate at 200–250 mg/mL of 100 mg/L stock solution used is generally suitable; any cloudiness that develops in this bath should disappear with agitation.

A cheaper alternative to tricaine is *2-phenoxyethanol*, an oily liquid that dissolves in water with shaking and is also used as an antibacterial, antifungal bath. 2-Phenoxyethanol produces anesthesia at 200–500 ppm, and remains effective as a working solution for several days. Some people have reported skin irritation after putting their bare hands in anesthetic baths of 2-phenoxyethanol.

Two newer or less-used anesthetics for fish that are excellent alternatives to tricaine and 2-phenoxyethanol are *metomidate hydrochloride* and *benzocaine*.

Metomidate hydrochloride (Marinil®; Hypnomidate®), now being registered as an anesthetic for food fish in Canada and the USA, is a highly soluble analogue of the medical anesthetic etomidate. Its main advantage is extremely rapid action at low concentrations (5–20 ppm), and it can be very effective when squirted directly into the mouth of a fish that has been cornered. It is also one of the few fish anesthetics that is also effective when injected intramuscularly. Recovery time is, however, longer than with tricaine or 2-phenoxyethanol. Metomidate also has promise as a long-term sedative at lower concentrations for transport of fish, and should be considered as a tool for limiting stress when broodstock must be shifted from one location to another. No information is yet available on irritation to humans.

Benzocaine is chemically related to tricaine but is cheaper and less stressful to fish because it is neutral in solution. It must, however, be dissolved in ethanol (100 g/L) to form a stable stock solution; it is used at about the same concentration as tricaine. Benzocaine is also available as the water-soluble hydrochloride salt, but is irritating to fish in this form because of the low pH of the resulting anesthetic bath, and solutions of the drug must be chemically neutralized. Information on irritation to users is not available, but the drug is commonly used as a topical human anesthetic (in throat lozenges, for example).

Methods of hormone administration

Spawning hormones are administered at present by two general methods: *injecting* a water or saline solution or *implanting* a slow-release pellet. Traditionally, both have been done either *intramuscularly* (IM) or *intraperitoneally* (in the abdominal body cavity, or IP;

Fig. 8). Oral (in the food) and topical (from the water) uptake of some hormones have been shown experimentally; although these routes are not yet practical, they offer intriguing advantages and should be tried on a larger scale.

Injection

Some workers choose to inject IM, and some to inject IP, without any justifications other than tradition. No controlled studies with fish justify choosing one technique over the other. Whatever the route of injection, it should not be varied if consistent results are desired.

One advantage of IM injection is standardization; it is easy to inject at the same spot and to the same depth in many fish. Using the finest possible needle leaves the smallest possible hole for the solution to leak out through and, in large fish, the muscle mass is thick

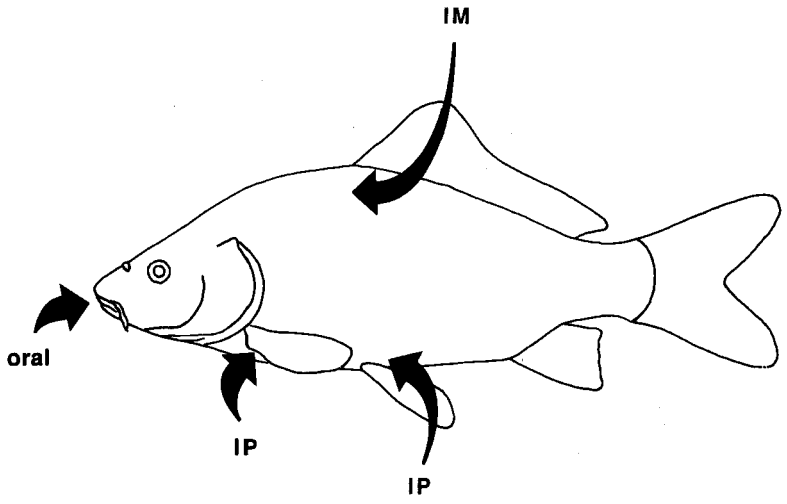

Fig. 8. Three routes for administering spawning hormones to fish. Intramuscular (IM) and intraperitoneal (IP) may be by injection or slow-release implant.

enough to allow placing the liquid deeply. It also helps to keep injection volume as low as possible, and to withdraw the needle slowly after waiting a few seconds for the liquid to find its way between muscle fibres. A good general rule is to keep volume under 0.5 mL/kg, and to inject in several locations if the total is much greater than 1 mL.

IP injections are often tolerated by unanesthetized fish, although an IP injection into the body cavity can easily end up in the intestine or gonad, and drug absorption is hard to standardize without careful needle placement. Experience with a given species helps to alleviate this problem. Larger diameter needles can be used in IP injections, facilitating the injection of suspensions of pituitary powder or domperidone and of more viscous solutions, such as Ovaprim®. Larger volumes (2–3 mL/kg) can also be used. IP injection should be done behind the pelvic or pectoral fin.

The number of injections naturally varies with the hormone used and the state of readiness of the animal. Methods that work with a single injection — GnRHa–dopamine antagonist mixtures frequently do — have the great advantage of minimizing handling; in excitable species (particularly marine ones), the extra stress of a second capture may cancel out the benefit of a second injection.

Implantation

Drugs injected after dissolving them in saline solution enter the general circulation within minutes and are then metabolized and excreted. In some special cases, it is useful to make the hormone available to the animal over weeks or months, and this can been done by mixing the drug with a binding material from which it is slowly released (*silicone rubber* or *Silastic*®, normally

used to deliver steroids) or which slowly breaks down allowing the hormone to escape (*cholesterol* or *cholesterol–cellulose*).

Although implants work well in some situations, they are not commercially available in sizes and doses for fish culture, and must be manufactured by hand according to published methods (see references for methods of making cholesterol or cholesterol–cellulose pellets, the only type feasible to manufacture in the laboratory). The special equipment required and the need to handle relatively large quantities of powdered hormone so far make this technique impractical except for research. Hormone dosages for all forms of implant exceed those used for injection at present and, if fish have already reached the stage of final maturation, injection is usually preferred.

Silicone or cholesterol–cellulose pellets can be implanted intramuscularly or intraperitoneally. IM allows positioning of the pellet and release of the drug can be standardized, although the muscle mass must be great enough to accommodate the pellet. An antibiotic cream should be applied to the IM implantation site to reduce the chance of infection.

Dietary administration

It is widely believed that, because many polypeptides and proteins are ineffective when taken orally by humans, the fish gut must similarly degrade protein hormones in the diet. This is almost certainly an oversimplification because enzymatic degradation of proteins varies with species and diet; many fish, including the large cyprinid family, are *agastric* ("stomachless"), and drug absorption in these species does not follow the mammalian pattern. Oral delivery of relatively stable GnRHa has the great advantage of

eliminating handling stress, a factor that makes many induced-breeding procedures fail.

Recent studies with several cold-water species point the way to a third alternative to injection and implantation of GnRHa. In sablefish, a handling-sensitive fish that responds to injected GnRH, oral intubation (tube feeding) of the hormone at 1 mg/kg resulted in spawning (Solar et al. 1990). Experiments with species of sea trout show that mammalian GnRHa can be absorbed from the fish gut when present in the food and, although 10 times as much hormone was used as with intramuscular injection, the oral treatment did induce ovulation and spontaneous spawning (Thomas and Boyd 1989).

Much work needs to be done to find the lowest effective doses and to determine whether the technique works for long-term administration as well as for final maturation and ovulation; however, the way is certainly open for some interesting experiments. When bulk prices for GnRHa and the savings in labour and broodstock are considered, the oral method may prove highly cost-effective. In Taiwan, at least one milkfish farmer is mixing GnRHa with broodstock diet and obtaining good spawning success.

Techniques for artificial fertilization

Fish that have been induced to ovulate and spermiate with hormones are often *strip-spawned*; that is, the gametes are removed by gently compressing the abdomen and then combined artificially. With more and more emphasis being put on environmental manipulation as a way of inducing maturation, natural spawning in enclosures is becoming more important (and

culturists are having to think about mechanical solutions to the problem of egg collection). Nevertheless, artificial fertilization has unique advantages and will continue to be used in many situations. Some of these advantages are that:

- Spawning enclosures are not required;
- Handling of fertilized eggs is easier;
- Milt can be used efficiently when it is scarce (by dilution or preservation);
- Mixing of gametes for genetic improvement is easier; and
- Interspecific or intergeneric hybrids can be produced.

Fish vary enormously in gamete characteristics: some eggs are sticky and some are buoyant; some are less than 1 mm across whereas others are five times as large. Artificial mixing of eggs and spermatozoa can produce very high fertility — over 90% — but techniques vary from fish to fish, and have to be worked out by trial and error.

Spermatozoa are kept immotile (unmoving) in the testis by high concentrations of potassium. When they are shed into the surrounding water, the potassium is diluted and the sperm cells are activated (start to swim or become motile). Motility usually lasts less than 1 minute, and fertilization itself requires only a few seconds of contact between eggs and spermatozoa. To complicate matters further, the eggs of many species are activated by contact with water and must be fertilized immediately.

The *dry method* is the best basis for a fertilization technique, because it takes advantage of these aspects

of gamete physiology. To make the most out of the very brief period of sperm motility, mixing with eggs should be done before any water is added. If much ovarian fluid accompanies the eggs, sperm cells will start to swim upon dilution in this liquid anyway, and adding further water to the eggs will only increase the percentage of motile cells. The best procedure, then, is to mix sperm with eggs, quickly add just enough of the natural spawning water to wet them thoroughly, and wash with larger volumes of water after several minutes.

This simple method could be greatly improved and, as researchers learn more about sperm physiology, they will be able to develop diluents that are mixed with sperm and prolong its motility so that fertilization can be done in one simple step. Solutions that eliminate egg stickiness in fishes like carps are also useful (Woynarovich and Horvath 1980).

Most fish farmers use vastly greater quantities of sperm than are needed to fertilize a given number of eggs. It is an indication of how poorly worked out these methods are that salmon farmers, who have access to the latest fish-farming technology, still use much more milt than is necessary for fertilization.

Using gonadotropins to induce breeding

Hypophysation

Fish breeders have been practicing *hypophysation* — injection of crude fish pituitary extracts — since the 1930s, and the technique needs no introduction here. Hypophysation is simple replacement therapy: gonadotropin from another fish takes over when the breeding fish is not producing enough of its own, and

triggers the remaining links in the hormonal sequence leading to gamete maturation.

Hypophysation has many advantages and many drawbacks. Now that GnRH analogues are available as a standardizable alternative, fish breeders can afford to look closely at the disadvantages of using pituitaries. The reason hypophysation works so well in some cases is also the reason it is hard to standardize: extracts are highly impure, and contain accessory hormones and other components that may stimulate some fish but inhibit others. Because the number and amount of these components in a given pituitary are unknown, their action is unpredictable.

A typical modern hypophysation technique for freshwater fish involves two injections into females: a small dose stimulates germinal vesicle migration, and is followed about 12 hours later by a larger one that induces germinal vesicle breakdown, ovulation, and spawning (Woynarovich and Horvath 1980). Males are generally injected only once, at the time of the female's second injection, to induce sperm hydration coinciding with ovulation in the female. Pituitary homogenates are also used in combination with other hormones (usually GnRHa) and often have a potentiating effect.

Most experienced fish breeders agree that, if good pituitaries are available, hypophysation is an excellent method of spawning fish. However, this judgment hinges on the implications of "good" and "available." Let us look at these.

Availability of pituitaries

Fish pituitaries are commercially available from many sources. Several companies offer whole or powdered carp and salmon pituitaries in dried form. At

greater cost, extracts with stated gonadotropin activity are available that could be used to standardize the technique. Small-scale farmers typically collect the pituitaries themselves from mature, often unrelated fish, and preserve these either in alcohol or dried, after acetone extraction of fats. Both sources of supply are costly in money or time, because both are labour-intensive and involve collecting large numbers of tiny glands when the donor fish are ripest. Collecting indigenous pituitaries is not necessarily an advantage. In Brazil, for example, the country where hypophysation began, several important species (*Piaractus* and *Colossoma*) respond well to hypophysation with *Prochilodus* pituitaries, but the time and effort involved in collecting these at the spawning grounds is enough to make local culturists look for a more reliable alternative.

Specificity and stability of pituitaries

Because gonadotropins are proteins, they are more or less species specific, and if pituitaries come from a species different from the one being spawned, the dosage may be different. Nevertheless, pituitaries from common carp are widely used to spawn a variety of species and have been described as "universal donors."

Instability of extracts is also a major contributor to variable results. First, the history of locally collected and stored pituitaries is frequently hard to verify. They may, for example, have been frozen and thawed before extraction and storage — and even very short warming of crude biological material virtually guarantees rapid enzymatic degradation. The same problem exists for solutions or suspensions of pituitaries: with the best rule being to use these preparations immediately and avoid storage altogether. If pituitaries must be stored, they should first be defatted in several changes of cold

commercial-grade acetone over 24 hours, then dried at ambient temperature and stored in a sealed vial in the dark. Acetone-dried pituitaries are commonly stored for several years, although there are reports of declining activity in inducing ovulation or in the volume of eggs produced. None of this information is quantified. Again, the best course is to use pituitaries as soon as possible. An alternative to acetone drying is to freeze pituitaries immediately on dry ice (–79°C); however, pituitaries frozen this way must be stored below –20°C and preferably below –40°C.

The varying potency of crude pituitary preparations also means that different groups working on the same species cannot compare results. Two pituitaries may look identical and yet be vastly different in gonadotropin content. The more mature the donor, the higher is the gonadotropin content likely to be. When culturists collect pituitaries themselves, some control can be gained by using only donors that look mature. Sex of the donor is not important, as long as the donor is mature. One way to get around variability in potency is to pool a large number of glands and use an aliquot of the extract for injection into each fish.

Partially purified fish gonadotropin

The problem of unstandardized gonadotropin content in crude pituitary homogenates can theoretically be overcome by purifying the material. *Salmon gonadotropin* (SG G100), partially purified and standardized by bioassay, is still available commercially and does have the advantage of long storage life. Its high cost limits its use to research, however, and in practice it offers no advantage over high quality crude pituitary preparations.

Mammalian gonadotropin

Human chorionic gonadotropin (HCG) is produced by the placenta and can be extracted from urine. HCG is available from many suppliers as a pharmaceutical drug, and has the advantages of purity and long storage life when frozen.

The problem with using HCG in fish culture is that the molecule is so unlike fish gonadotropin that high doses must be used for many species and some may not respond at all. Also, because HCG is not cheap to begin with, the method is often not economical. HCG works well for some species — including marine fish — usually in combination with crude pituitaries or GnRHa, and the cost can be reduced by extracting the hormone locally, as has been done in Thailand.

The possibility of an immune reaction in fish repeatedly spawned using a pure protein like HCG has often been raised, but recent research using a highly sensitive assay for HCG antibodies in silver carp and goldfish has shown that the problem may be much exaggerated, and that poor reproductive performance in broodstock repeatedly injected with HCG results from other factors (Van der Kraak et al. 1989).

Using releasing hormones to induce breeding

New users of GnRH methods are confronted by a large assortment of releasing hormone analogues and terminology. Let us try to bring some order to this confusing area.

GnRH analogues

Mammalian GnRH (also called LHRH) was purified and synthesized in the early 1970s. The molecule has 10 amino acids[1] joined together, with an *amine group* (NH_2) attached to position 10:

Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

Salmon GnRH was purified about a decade later. The only difference from mammalian GnRH is that amino acids 7 and 8 are tryptophan and leucine:

Glu-His-Trp-Ser-Tyr-Gly-**Trp-Leu**-Pro-Gly-NH_2

This minor change over 400 million years of evolution reflects the importance of the basic molecule in reproduction in *all* vertebrates, and is the reason mammalian GnRH analogues are usually just as effective for induced reproduction as those from fish.

Because GnRH molecules are simple, they can be synthesized, and different amino acids can be inserted at any position to produce artificial analogues that are characteristically 50 to 100 times as potent (*superactive*) as the natural form. Many analogues of mammalian and fish GnRH have been made, often leaving out amino acid 10 entirely. For LHRH alone, where a superactive and long-lived analogue is potentially worth a great deal of money as a pharmaceutical drug (it is at present used in treatment of prostate cancer and ovarian fibroids and also has antifertility effects when given chronically), more than 2 000 analogues have been synthesized.

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[1] Ala — alanyl; Arg — arginyl; Glu — glutamyl; Gly — glycyl; His — histidyl; Leu — leucyl; Pro — prolyl; Ser — seryl; Trp — tryptophanyl; Tyr — tyrosinyl.

Two analogues useful in fish culture are an analogue of mammalian GnRH, called *LHRHa*

Glu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-NH-CH_2-CH_3

and an analogue of salmon GnRH, called *sGnRHa*

Glu-His-Trp-Ser-Tyr-D-Arg-Trp-Leu-Pro-NH-CH_2-CH_3

D-Amino acids, which are mirror image forms of the naturally occurring L-forms, have been substituted at position 6, making the molecule much more resistant to degradation in the body and therefore longer-lasting. Both analogues lack the 10th amino acid, and instead end with an *ethylamide* (NH-CH_2-CH_3; abbreviated to NEt).

GnRH analogues are given shorthand names based on the parent molecule; unfortunately, manufacturers and authors vary the terminology. The proper name for the mammalian GnRHa above is [D-Ala^6, Pro^9 NEt] LHRH. The analogue of salmon GnRH should be called [D-Arg^6, Pro^9 NEt] sGnRH. The term "des Gly^{10}" means "no Glycine at position 10," and is often used in place of "Pro^9".

Advantages of releasing hormones

Releasing hormones have three great advantages over gonadotropins for inducing maturation and spawning. The first two are biological: they act early in the hormonal chain and cause the fish to produce its own gonadotropin, thereby eliminating all the problems caused by using a gonadotropin from another species. Second, the GnRH molecule itself is not highly species specific. Third, they are simple, easily manufactured, stable molecules whose biological activity does not vary from lot to lot and, because they are active at such low concentrations, their use is economical.

Early trials of GnRHa in induced breeding included some dramatic successes but many failures as well, and it is only in the past few years that reliable methods for many species have begun to appear. For some species, GnRHa is highly effective on its own; for others, particularly carps, it is much more effective when supplemented by a dopamine antagonist. The cost of treatment is still dropping as more manufacturers offer the hormones for aquaculture, and GnRH-based methods are likely to be the backbone of induced reproduction while the next generation of technology — methods based on environmental manipulation — is perfected.

Choice of GnRH analogue for induced reproduction

Many GnRH analogues, both mammalian and fish, are highly effective inducers of gamete maturation, ovulation, and spermiation in fish. There may indeed be an overall "best" analogue, but variation in species response, differences in state of maturity at time of administration, and the degree of dopamine inhibition of gonadotropin release make it impossible to recommend one analogue over another. Often a slight increase in dosage makes up for lower activity in a given species, and varying the dose is more productive than worrying about which analogue to use. Some analogues are more expensive to make (i.e., synthesis is more complicated) or sell (i.e., royalty fees must be built into the cost), and because cost-effectiveness is so important in choosing an analogue it often makes more sense to use more of a less potent, cheaper one.

Salmon GnRHa closely resemble GnRH in all cultured fishes, and some studies show better binding to pituitary receptors and better release of gonadotropin than is achieved with mammalian GnRHa. However,

there is no practical superiority of [D-Arg^6, Pro^9 NEt] sGnRH (the most commonly available one) over commonly available analogues of mammalian GnRH such as [D-Ala^6, Pro^9 NEt] LHRH. Fish culturists should certainly take the lead from published reports of the use of GnRHa in their species, but when cost — sGnRHa is more expensive than mammalian — and easy availability are factors (as they usually are), analogues of mammalian GnRH are perfectly adequate. The fact that GnRH is only the "on" part of an "on–off" control system for gonadotropin release means that the analogue one uses may be less important than whether it is accompanied by a dopamine inhibitor.

Availability and stability of GnRH analogues

Several suppliers offer dry powdered synthetic analogues of mammalian GnRH for sale to fish culturists. All are effective. Some are mixed with an inert binder that makes handling of small quantities of hormone easier. Synthetic salmon GnRHa is, at present, marketed only by one company in a formulation with the dopamine inhibitor domperidone (Ovaprim®; Syndel Laboratories Ltd).

All the available GnRH analogues are much more resistant to enzymatic breakdown than the parent molecule. Solutions of these analogues are theoretically stable indefinitely as long as they stay sterile; in practice, however, it is seldom easy to use a sterile needle for each injection, and the best policy is to make up the minimum volume every few days during the spawning season. If dissolved GnRH must be stored, it should be frozen as a concentrated stock solution in sterile distilled water.

GnRH analogues are potent pharmaceutical drugs,

and should be handled carefully. Their action in humans is similar to that in fish — stimulation of gonadotropin and sex-hormone release — with the important difference that chronic administration in humans leads to a decrease in gonadotropin release and therefore in sex steroids. (This is why the drug can be used to treat cancer of the prostate, which responds to a lowering of testicular androgens.) There is very little chance a fish culturist could receive a sustained dose of the drug, and reasonable precautions (avoiding contact and inhalation) are adequate. GnRH should not be handled by pregnant women, and fish treated with the drug should not be eaten.

GnRH has also been formulated, although only for experimental purposes, as a slow-release pellet or implant, usually in a matrix of cholesterol or cholesterol–cellulose. These pellets must be hand-made and, although they can work impressively, particularly to synchronize and advance early maturation, few culturists will have the equipment to make them. GnRH implants work best when fish are not completely ripe, and the slowly released hormone can influence gonadotropin secretion over an extended period. Implanting a GnRH pellet in a ripe fish is a waste of hormone because all that is required is a one-time elevation of plasma GnRH, and that can be accomplished by injection.

Using a GnRH analogue together with a dopamine antagonist

In the early 1980s, gonadotropin secretion in goldfish was shown to be regulated by a dual neuro–hormonal system: GnRH stimulates, dopamine inhibits. The system was first identified by Canadian researchers and later collaboration with colleagues in China

demonstrated it in economically important cyprinids. It is not yet known whether gonadotropin is regulated by this "on–off" mechanism in all teleosts, and dopamine inhibition may be weak or even nonexistent in some species.

Inhibition by dopamine can be blocked by injecting a drug that specifically ties up dopamine receptors, or one like *reserpine* that generally depletes all catecholamines (one of which is dopamine). The action of reserpine is so general and the drug has so many side effects that it should not be used in fish culture.

Two specific dopamine antagonists, however, are useful. These are *pimozide* and *domperidone*. Domperidone is cheaper and more potent than pimozide and has the advantage of not crossing the blood–brain barrier, so this drug is at present the recommended one. Domperidone is widely prescribed in human medicine as Motilium® (Janssen Pharmaceuticals) to modify gastrointestinal motility. Because the list of dopamine antagonists is long, we can expect other drugs to be effective in combination with GnRH; whether these will be useful to fish breeders depends on their cost and availability.

Linpe method

Injecting a GnRHa followed by (or in combination with) a dopamine antagonist has been called the *Linpe method*, after Lin and Peter the researchers who started it. Most of the work resulting in the Linpe method was done on cyprinids, and there is convincing evidence for these fish that the method is effective where injection of GnRH alone is not.

It is tempting to generalize about the superiority of the Linpe method for *all* cultured fish, but because

comparative experiments under field conditions — GnRHa–domperidone versus GnRHa alone — have only been done for carps, it is still too early to do this. Some researchers have tried to put fish in categories that reflect the strength of dopamine inhibition, ranging from cyprinids (strong dopamine effect) to salmonids (weak dopamine effect). The danger of making this kind of list relates to what we have already said about differences in the effect of GnRH itself: factors such as the general readiness of the fish can outweigh any advantage or disadvantage of a particular treatment.

The commonly voiced view that marine fish do not require domperidone along with GnRHa requires more proof; in milkfish and mullet, for example, two of the most important warm-water marine species, there are no published reports of its having even been tried.

Until use of the Linpe method is more widespread, we will avoid such lists. In species that become fully sexually mature in captivity and respond to GnRHa readily — many salmonids fall into this category — a dopamine antagonist is not needed. In other species — the best evidence is still from cyprinids — even though they will spawn with GnRHa alone, delay to ovulation is shorter and more predictable when domperidone is added. Administering the two drugs is easy, with a single injection of a mixture being as effective as two separate injections. This has led to the manufacture of a commercial GnRHa–domperidone spawning "kit" that combines the two in a single solution (Ovaprim-C®). Enterprising fish farmers can of course always opt, as in Thailand, to buy GnRHa and domperidone as over-the-counter pharmaceuticals, and reconstitute them for injection into fish.

Doses of GnRH and domperidone

Although there will never be a standard method for spawning all species, culturists working with a single species can standardize methods by systematically eliminating sources of variability and using the lowest effective dose. Effective doses of GnRHa and domperidone vary widely and are not comparable because of differences in species, temperature, state of maturity, and GnRHa. The trend is toward single injections and, although GnRHa doses between 1 and 100 μg/kg have been effective, culturists should aim for the 5–20 μg/kg range. Domperidone is usually effective at doses of 1–5 mg/kg.

To facilitate economical use of GnRHa, without the need for tedious weighing of tiny amounts, it is best to buy preweighed small amounts of the hormone (e.g., 0.5 or 1 mg aliquots) and prepare a concentrated stock solution (e.g., 1 mg/mL) in sterile water in the original container. Appropriate amounts of a more dilute solution in 0.7% NaCl can then be prepared at the time of injection. GnRHa is most stable as a dry powder, but the sterile stock solution can also be kept for several months if frozen.

Domperidone and pimozide are not readily soluble in water and are sensitive to oxidation. They are best used as a suspension in 0.7% NaCl containing 0.1% metabisulphate as antioxidant, or can be dissolved (and injected) in propylene glycol. Commercially available domperidone tablets for humans (Motilium®) have been powdered, dissolved in propylene glycol, and used uccessfully in induced reproduction of fish (Fermin 1991).

Cost-effectiveness of GnRH analogue, fish pituitaries, and HCG

Businesses use the term *cost-effectiveness* to describe the balance between the cost of a product or process and how well it works. Often the balance is a compromise, but it must always be considered. In hormone-induced breeding of fish, effectiveness is more easily defined than cost, because cost really includes not just the monetary value of the hormone but the ease of obtaining it as well. It is also worth remembering that the cost of a spawning hormone may only represent a small percentage of the overall cost of fingerling production.

The three hormone types just described — fish pituitary gonadotropins, HCG, and GnRHa with or without a dopamine antagonist — are all effective in many situations, and we have seen that this effectiveness is often as much influenced by species and physiological condition as by hormone and dose. However, if several different hormonal approaches will work with a given animal — and there are many studies that show this — then one must be chosen, and the cost of each will be a major factor.

Of these three options, GnRHa is potentially cheapest because it is a simple molecule that is easily synthesized; prices will drop even further as more suppliers enter the market. Gonadotropins from fish and humans are expensive because they must be extracted from biological material that is labour-intensive to collect.

In all practical trials where *actual successful spawning dosages* have been compared for a particular fish (as opposed to a simple comparison of the cost of the raw materials), GnRHa has proven significantly cheaper. With mullet, for example, which can be spawned with

HCG or GnRHa, the actual cost per breeder in 1987 was 10 times higher for HCG (Lee et al. 1988).

Comparative cost-effectiveness with fish pituitaries is hard to judge because the potency of pituitary gonadotropins is uncontrolled, and labour costs vary widely from country to country. There are many cases, particularly in carp culture, where hypophysation will and should remain the mainstay of hormone-induced breeding when pituitaries can be collected locally. When acetone-dried whole or powdered pituitaries are obtained commercially, effective doses cost about 10 times as much as an effective GnRHa treatment.

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Chapter 4

Induced reproduction in some important cultured fishes

Experiments on hormone-induced reproduction in cultured fish are reported at the rate of several hundred each year. Many deal with cold-water species or warm-water sport-fishing species, but even listing those that deal with warm-water fishes cultured in developing countries would be a formidable task (and probably an unproductive one for culturists, because many of the papers would be impossible to obtain in their part of the world). For carps and catfish especially, the literature is very large, and instead of reviewing or even listing all these papers, we have taken a "case study" approach. Having made some general points about fish endocrinology, hormones used in induced breeding, and some other factors that affect breeding success, we now summarize selected published papers that illustrate these points.

Carps

Successful hormone-induced breeding of common carp (*Cyprinus carpio*), Chinese carps (*Ctenopharyngodon*

idellus, *Hypophthalmichthys molitrix*, and *Aristichthys nobilis*), Indian carps (*Labeo* spp, *Cirrhinus mrigala*, and *Catla catla*) and Thai carp (*Puntius gonionotus*) have been widely reported for more than a decade. The present trend is toward standardization and reduction of cost, so that, although many reports still appear in which pituitaries or HCG have been used, more and more studies are convincingly showing that GnRH analogues, particularly when combined with a dopamine antagonist (the Linpe method), are cost-effective. Hypophysation techniques for carps have been well worked out by Woynarovich and Horvath (1980). Recent field trials in India with the Indian and Chinese carps using Ovaprim-C®, a commercial preparation of sGnRHa and domperidone, have shown the method is highly effective.

A second trend is to extend these results to related species such as bream (*Parabramis pekinensis*) and black carp (*Mylopharyngodon piceus*).

The papers summarized below have been chosen either as examples of good experimental approaches or to illustrate the geographic range of studies on carps. Geographic range is important in carps because some species can be cultured in quite different climates and at different latitudes — Chinese carps in Moscow and Malaysia, for example — and varying culture conditions and ages at maturity make standard methods impossible.

Case studies

Species and broodstock details:
Bighead carp (*Aristichthys nobilis*), silver carp (*Hypophthalmichthys molitrix*), Thai carp (*Puntius gonionotus*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), and grass carp (*Ctenopharyngodon idellus*); Thailand; May–August; 3–5 females per treatment.

Assessment of gonadal state:
External appearance.

Treatment and results:
Bighead carp and silver carp: LHRH analogue ([D-Ala^6, Pro^9 NEt] LHRH) in distilled water was injected IM in two doses (5 and 15 µg/kg), separated by 18–20 hours, and produced 100% ovulation after 4–8 hours. Number of eggs was normal but fertility was not tested. By comparison, in trials with similar dosages and administration in the other carp species, LHRH analogue was ineffective alone, and had to be combined with fresh silver carp pituitaries. This lack of effect was suggested to be due to dopamine inhibition, and the authors recommend further studies with a dopamine antagonist.

Reference:
Ngamvongchon, S.; Pawaputanon, O.: Leelapatra, W.; Johnson, W.E. 1988. Effectiveness of an LHRH analogue for the induced spawning of carp and catfish in northeast Thailand. Aquaculture, 74, 35–40.

Species and broodstock details:
Grass carp (*Ctenopharyngodon idellus*) and bighead carp (*Aristichthys nobilis*); Sri Lanka; female and male broodstock 3–5 years old weighing 4–8 kg; spawned in previous years.

Assessment of gonadal state:
External appearance.

Treatment and results:
LHRH analogue (not identified) in distilled water was injected IP twice in females and once in males. Eggs and sperm were stripped for dry fertilization. For grass carp, methods combining a first injection of 20 μg LHRHa per fish with a second injection 12 hours later of 40–50 μg LHRHa and 0.5–1.5 mg carp pituitary was as effective as divided doses of carp pituitary alone in producing fertile eggs and postlarvae. Both males and females were induced to breed three times between April and October. In bighead carp, LHRHa was injected alone and in combination with 300–800 IU HCG and resulted in spawnings in April and July. Fecundity indicates that both species of carp spawned completely in each cycle.

Reference:
Kumarasiri, W.S.A.; Seneviratne, P. 1988. Induced multiple spawnings of Chinese carps in Sri Lanka. Aquaculture, 74, 57–62.

Species and broodstock details:
Common (*Cyprinus carpio*), silver (*Hypophthalmichthys molitrix*), mrigal (*Cirrhinus mrigala*), grass (*Ctenopharyngodon idellus*), bighead (*Aristichthys nobilis*), black (*Mylopharyngodon piceus*), and Thai (*Puntius gonionotus*) carps; various experimental and field stations in China; some broodstock previously spawned.

Assessment of gonadal state:
Not stated.

Treatment and results:
Summary of the Linpe method using LHRH ([D-Ala6, Pro9 NEt] LHRH) and salmon GnRH analogues with dopamine antagonists domperidone and pimozide. For all but mud carp and black carp, single injections of dopamine antagonist (1–5 mg/kg) with LHRH or GnRH (10–100 mg/kg) stimulated ovulation after 10–16 hours. Mud carp and black carp required a second injection of LHRH analogue. The authors consider the basic Linpe method to have advantages over traditional techniques (cost, standardization, and less handling) and suggest refinement using salmon GnRH and domperidone.

References: Research and field trials reported in several journals; review articles include:

Peter, R.E.; Lin, H.R.; Van der Kraak, G. 1988. Drug/hormone induced breeding of Chinese teleosts. *In* Idler, D.R.; Crim, L.W.; Walsh, J.M., ed., Proceedings of the 3rd International Symposium on Reproductive Physiology of Fish, St John's, Newfoundland, August 2–7, 1987. Marine Sciences Research Laboratory, Memorial University of Newfoundland, St John's, NF, Canada. Pp. 120–123.

Peter, R.E.; Lin, H.R.; Van der Kraak, G. 1988. Induced ovulation and spawning of cultured freshwater fish in China: advances in application of GnRH analogues and dopamine antagonists. Aquaculture, 74, 1–10.

Species and broodstock details:
Mrigal (*Cirrhinus mrigala*); India; 15 fish.

Assessment of gonadal state:
Not stated.

Treatment and results:
LHRH analogue ([D-Ala^6, Pro^9 NEt] LHRH) was tested in single injections at 5 and 10 μg/kg. No spawning was observed in controls and low dose LHRH; at 10 μg/kg LHRH, 120 000 ova/kg were stripped with 80% fertility and 70% hatching.

Reference:
Kaul, M.; Rishi, K.K. 1986. Induced spawning of the Indian major carp *Cirrhinus mrigala* with LHRH analogue or pimozide. *In* Billard, R.; Marcel, J., ed., Aquaculture of Cyprinids. Institut National de la Recherche Agronomique, Paris, France. P. 167 (abstract only).

Species and broodstock details:
Rohu (*Labeo rohita*), Thai carp (*Puntius gonionotus*), mrigal (*Cirrhinus mrigala*), silver carp (*Hypophthalmichthys molitrix*), and grass carp (*Ctenopharyngodon idellus*); northeast Thailand; April–October.

Assessment of gonadal state:
External appearance.

Treatment and results:
Field trials, at several fisheries stations, were done with several variations of the GnRHa–domperidone method. All were single IM injection of combined GnRHa–domperidone at various dosages (GnRHa 2–20 μg/kg; domperidone 2–10 mg/kg). Treatments included sGnRHa–domperidone prepared on site (252 fish) or as ready-formulated Ovaprim-C® (55 fish) applied at the height of the spawning season, and mammalian LHRH analogue ([D-Ala^6, Pro^9 NEt] LHRH)–domperidone applied late in the spawning season (86 fish). Fish typically spawned naturally in tanks after an overnight waiting period, with high rates of spawning and fertility in all treatments. Spawning success was higher with sGnRHa (mixed on site or as Ovaprim-C®), but the authors caution that trials using LHRHa in place of sGnRHa were done outside the normal spawning period. The authors stress the costs of all treatments: LHRHa–domperidone is about the same cost as hypophysation, but all sGnRHa formulations cost twice as much or more. The method has since been extended to several commercial farms in Thailand.

Reference:

Leelapatra, W. 1988. Carp culture in Thailand with particular emphasis on induced spawning. *In* Proceedings of the Aquaculture International Congress and Exposition, Vancouver, BC, 6–9 September, 1988. Pp. 331–337.

Catfish

Catfish is a desirable food fish that tolerates crowding and occasional periods of low dissolved oxygen. Practical methods for inducing ovulation and spawning in species of *Clarias* (including *C. macrocephalus*, *C. batrachus*, *C. gariepinus*, and *C. fuscus*) and *Pangasius* (*P. sutchi* and *P. pangasius*) in Southeast Asia and India have, until recently, relied on hypophysation and HCG. Although HCG gives acceptable results, using a GnRHa will reduce costs, and recent studies show that this method is effective. The next few years will see expanded field trials with GnRH analogues, particularly combined with a dopamine antagonist.

Catfish are also responsive to temperature manipulation for advancing ovarian maturation. Some *Clarias* species can be bred year round, with the greatest success in repeat spawning in locations where temperature is most constant.

The African catfish, *Clarias gariepinus*, has been the object of intensive study at Wageningen Agricultural University and the University of Utrecht in the Netherlands, and results have been applied in African pond culture. Culturists interested in breeding this fish should consult the summary of this work in the special issue of *Aquaculture* (Volume 63, 1987).

Readers interested in breeding of the channel catfish, *Ictalurus punctatus*, should refer to Tucker (1987).

Case studies

Species and broodstock details:
Thai catfish (*Clarias macrocephalus*); Malaysia.

Assessment of gonadal state:
External appearance.

Treatment and results:
A single IM injection of HCG, 2 000 IU/kg at 26–31°C was used. Ten fish were strip-spawned 10 hours later, with average hatching of 73% after incubation at 30°C. (The same basic method has been used for Chinese catfish (*C. fuscus*) in Hawaii, although the dose of HCG was divided, and a doubling of the dose was found to improve response at the beginning and end of the spawning season).

Reference:
Mollah, M.F.A.; Tan, E.S.P. 1983. Viability of catfish (*Clarias macrocephalus*) eggs fertilized at varying post-ovulation times. Journal of Fish Biology, 22, 563–566.

Species and broodstock details:
River catfish (*Pangasius sutchi*) and Thai catfish (*Clarias macrocephalus*); Malaysia.

Assessment of gonadal state:
External appearance and size, colour, and position of nucleus of the eggs (obtained by cannulation).

Treatment and results:
Both species received IM injections of various hormones in 0.6% saline solution. *Clarias* was strip-spawned, whereas *Pangasius* spawned spontaneously. *Pangasius* females were treated with combinations of pituitaries, HCG, and LHRH analogue. Highest percentage spawning was after two doses of HCG (450 IU/kg each) combined with pituitaries of *Tachysurus*; spawning was also acceptable when LHRHa was combined with *Pangasius* pituitaries and delivered in two injections of between 10 and 20 μg/kg. Percentage hatching was comparable in the two treatments. LHRH analogue alone (divided doses of 20–50 μg/kg) was also effective, with spawning ranging from 33–100% (13 fish). In larger scale trials with *Clarias*, hormones were not combined and the entire dose was injected at once. HCG at 3 000–4 000 IU/kg was more effective that LHRH analogue at 10–30 μg/kg, but the promising results with LHRHa — 88% spawning in one group of eight fish — indicates that the technique will work with catfish.

Reference:
Saidin, T.; Othman, A.A.; Sulaiman, M.Z. 1988. Induced spawning techniques practiced at Batu Berendam, Melaka, Malaysia. Aquaculture, 74, 23–33.

Species and broodstock details:
Thai catfish (*Clarias macrocephalus*); northeast Thailand; May–August.

Assessment of gonadal state:
External appearance.

Treatment and results:
LHRH analogue ([D-Ala^6, Pro^9 NEt] LHRH) in distilled water was injected IM in a single dose of 20 µg/kg at 29–32°C. Ovulation and strip spawning followed within 18 hours with a success rate of 70%. Response to a single injection of HCG (4 000 IU/kg) was 100% ovulation. Although the numbers of fish treated were low (three for each treatment) and fertility or hatching rates are not stated, results with LHRHa confirm the effectiveness of this hormone in *Clarias*.

Reference:

Ngamvongchon, S.; Pawaputanon, O.; Leelapatra, W.; Johnson, W.E. 1988. Effectiveness of an LHRH analogue for the induced spawning of carp and catfish in northeast Thailand. Aquaculture, 74, 35–40.

Milkfish

Roughly 10% of the total world production of cultured fish is milkfish in Indonesia, the Philippines, and Taiwan. Milkfish are grown in brackish-water ponds and in net pens in lakes, and the fry requirement (2–3 billion per year in the Philippines alone) cannot be met by capture from the wild. Breeding milkfish in captivity has been a top research priority for the past decade with major financial backing from international donors including the International Development Research Centre and the US Agency for International Development.

Most of the work in hormone-induced spawning has been done at the Southeast Asian Fisheries Development Center (in the Philippines), the Tungkang Marine Laboratory (Taiwan), the Gondol Research Station (Indonesia), the Oceanic Institute (Hawaii, USA), and the University of Victoria and Memorial University of Newfoundland (Canada). As a result of these studies, captive milkfish can now be induced to mature and spawn using hormones. Both GnRH and HCG are effective; there are no standard procedures because broodstock are maintained differently (in tanks, sea pens, or ponds, with different diets and stocking densities) and mature at different ages.

Research on control of milkfish reproduction falls into one of three categories: *induced spawning*, *induced maturation*, and *spontaneous spawning*. Research on induced maturation reflects the inability of broodstock in some conditions to mature far enough for induced spawning to be effective, and involves chronic hormone treatment.

Hormone-induced spawning of milkfish

Recent work on hormone-induced spawning of milkfish has been the subject of several good reviews, including Marte (1988b), Marte et al. (1988a, b), and Lee et al. (1986). An average egg diameter of at least 660 μm (obtained by cannulation) was previously accepted by most workers as indicating the minimum gonad development for successful induced spawning with a single injection of HCG; best results with GnRHa and HCG are now obtained when vitellogenesis is completed and the average egg diameter reaches 750 μm, with a single size class of oocytes (Tamaru et al. 1988b).

A consistent feature of induced spawning trials with milkfish is the animal's susceptibility to handling stress. When milkfish are netted, anesthetized, tagged, and treated with hormones, mortality can be as high as 50%; obviously, methods that require little or no handling must be developed urgently. Natural spawning in enclosures after hormone induction is highly preferable to strip-spawning. (The ideal situation, of course, is spawning without any intervention at all — as long as timing is reasonably predictable — and this, in fact, is the present trend in milkfish reproduction in captivity.)

Marte (1988b) reviews recent results in artificial propagation of milkfish in the Philippines; and further details are provided in Marte et al. (1988b). Excellent results were obtained with sea pen-reared broodstock given single injections of HCG (1 000 IU/fish) or mammalian or salmon GnRH analogues at 10 or 100 μg/kg; the mammalian analogue [D-Ala^6, Pro^9 NEt] LHRH was as effective as salmon GnRHa and was cheaper and easier to obtain. Spawning was spontaneous, frequently multiple and, as would be expected in a fish so sensitive

to stress, occurred more readily in fish returned to sea pens than in those placed in tanks.

Successful spawning of tank-reared milkfish with LHRH analogue is described by Tamaru et al. (1988a). The broodstock used in these studies, in contrast to those held in net pens in the Philippines, had received chronic treatment with LHRH analogue and 17α-methyltestosterone to promote oocyte maturation to the stage where acute LHRH injection would stimulate ovulation. Percentage successful spawning increased as egg diameter exceeded 700 μm, with best results between 750 and 800 μm. Fish spawned naturally as long as males were injected along with females. The minimum effective dose of LHRH analogue ([D-Ala^6, Pro^9 NEt] LHRH) varied between 1 and 5 μg/kg, although the authors recommend 10–20 μg/kg for practical work. An important feature of these studies at the Oceanic Institute in Hawaii is that broodstock had been handled frequently for years and tolerated this disturbance much better than broodstock in sea pens.

Several studies have compared slow-release preparations of GnRHa (cholesterol–cellulose pellets or osmotic pumps) with single injections of the hormone. There is no advantage of sustained release as long as the fish have attained sufficient maturity, and injection is far easier to do.

Induced maturation

Like many marine species, milkfish held in tanks may not mature enough for an injection of GnRHa or HCG to stimulate spawning; if they do, it is often at a relatively advanced age. To accelerate maturation and promote rematuration in broodstock that have already spawned, some researchers have used slow-release

formulations of LHRH analogue and 17α-methyltestosterone, usually implanted intramuscularly as cholesterol–cellulose and Silastic-encapsulated pellets respectively. Implanting pellets is not a practical production technology because the pellets must be hand-made, but these studies do show that making GnRHa and testosterone available over a period of months can promote maturation. Tamaru et al. (1988a) applied LHRH analogue and 17α-methyltestosterone monthly to tank-reared milkfish in Hawaii and were able to substantially increase the number of mature females: 85% of implanted females completed vitellogenesis, compared to 33% of controls.

Maturation experiments in the Philippines are summarized by Marte et al. (1988a); results with chronic LHRH–testosterone therapy were less clear-cut than those from Hawaii because of the varied reproductive history of the experimental animals, but maturation was advanced in some 4-year-old tank-reared fish.

Spontaneous maturation and spawning

A decade of intensive research on induced spawning in milkfish has clearly shown that increased handling of broodstock in anything approaching a production situation brings diminishing returns. Induced spawning with GnRH analogues is successful in females with oocytes greater than 700 μm diameter but injected broodstock must be allowed to spawn naturally.

The next logical step is to provide the conditions in which milkfish mature and spawn *without intervention*, as has now been repeatedly demonstrated in sea pens, tanks, and ponds in Indonesia, the Philippines, Taiwan, and the USA (Hawaii). Reports of spontaneous spawning cover such varied holding conditions and climates

that there is an obvious opportunity to make more practical use of this phenomenon. To do so, breeders will have to elicit spawning at an earlier age — many natural spawnings have been in 6- to 12-year-old fish, which is not economic in a species that can spawn after 4 or 5 years — and they will have to develop better ways to collect fertilized eggs. Prospects for solving both problems are excellent.

Spontaneous spawnings in net pens (also called floating cages) in the Philippines were reported in the early 1980s and can now be relied on in pens of at least 6 m diameter. Net pens in the Philippines are round; nobody has tried holding milkfish in rectangular pens such as those used in salmon farming, and a different surface area may be needed with a differently shaped enclosure. The 6-m minimum size appears to apply to tank-reared fish as well. In 1987, there were 52 spontaneous spawnings out of a population of 100 5-year-old broodstock in these net pens, yielding 36 million eggs (Marte 1988a).

Repeated spontaneous spawnings have also occurred in large, land-based, pumped sea-water ponds in Taiwan, but broodstock were old (9–11 years). In 1984, for example, there were 62 spawnings, and 61 million eggs were collected. Several females spawned several times in one season (Lee et al. 1986). Spontaneous spawning in tank-reared milkfish has been reported in Indonesia and the USA (Hawaii), but is not as reliable as spawning in net pens and ponds. In Indonesia, 9- and 10-year-old broodstock spawned in holding tanks several months after transport from another facility, even though mortality during transport was 12% (Prijono et al. 1988).

It is clear from all these reports of spontaneous spawning that approximating natural conditions — as

in the floating net pens in the Philippines — is the best way to achieve spawning at the earliest possible age. Collecting fertilized eggs is still a problem and rearing the larvae to a salable size still needs to be worked out; but, when these problems are solved, the objective of providing adequate supplies of milkfish seed will have been reached through husbandry, not applied endocrinology.

Mullet

The mullets (family Mugilidae) are an important protein source in the Pacific Basin, Southeast Asia, India, the Mediterranean, and some parts of Central and South America. Mullet live in coastal areas and are euryhaline, so they can be grown in fresh, brackish, or sea water.

Reproductive problems in captive mullet resemble those in milkfish. Handling stress is a common problem and attempts to induce spawning in wild-caught broodstock have given way almost completely to studies with tank-reared fish. Many of the research groups working on milkfish have also worked on mullet. Most of the work has been done on *Mugil cephalus*, the grey (sometimes called striped) mullet.

Spontaneous spawning of captive mullet broodstock reared in tanks or ponds (mullet are not normally held in floating net pens) has not been recorded. Mullet mature less readily in captivity than milkfish, and many studies have been aimed at finding an effective *chronic* hormone therapy to provoke vitellogenesis and spermatogenesis.

Induced-spawning techniques have followed the milkfish pattern: partial success with salmon gonadotropin,

adequate but expensive methods using combinations of carp pituitaries and HCG, and more recent trials with LHRH analogue alone or in combination with pituitaries. A consistent feature is the relatively high doses of gonadotropins and GnRH analogues needed to induce spawning, although this may reflect less than optimal broodstock husbandry rather than an inherently poor response to the hormones. The future will almost certainly see more reliable results with GnRH analogues; also, although many people feel dopamine antagonists are unnecessary in marine fish, these compounds should also be tried.

Induced maturation and spawning in grey mullet

The state of the art in induced maturation and spawning in grey mullet (*Mugil cephalus*) has recently been reviewed by Lee and Tamaru (1988), and their paper includes a summary of research at the Oceanic Institute in Hawaii.

Maturation of both male and female mullet can be induced outside the normal spawning season by manipulating temperature and photoperiod, or by implanting slow-release cholesterol pellets or Silastic capsules containing 17α-methyltestosterone (for males) or LHRH analogue plus testosterone (for females). The form of the steroid used is important: unlike female milkfish, female mullet do not respond to chronic 17α-methyltestosterone treatment.

After induced maturation, female mullet can be spawned several times in a season using a variety of acute hormonal treatments. Although HCG is effective, it is also expensive at the doses needed, and trials with GnRH analogues (usually [D-Ala^6, Pro^9 NEt] LHRH) are

promising. One cost-effective method is to use 20 mg/kg carp pituitary followed by 200 μg/kg LHRH analogue; two injections of LHRHa are nearly as effective. (Readers of Lee and Tamaru's review (1988) should be alert for a consistent typographical error that expresses LHRH doses as **mg/kg** rather than **μg/kg**, multiplying the dose and cost a 1 000-fold!)

Induced maturation is explored in greater depth in Tamaru et al. (1989), with details on multiple spawnings in captivity where egg and larval quality were unimpaired. Females are considered fully mature and ready for induced spawning when oocytes reach an average diameter of 600 μm.

Other mullet species

Wild-caught broodstock of *Mugil liza* have also been induced to spawn, although a third of the females died during treatment (Alvarez-Lajonchere et al. 1988). Effective doses of HCG when used alone were high — 50 000–60 000 IU/kg — although great care was taken in characterizing receptive females by oocyte diameter (greater than 600 μm), appearance of the yolk, position of the nucleus, and a lack of visible oil droplets. HCG-induced natural spawning of pond-reared broodstock of the Mediterranean mullet (*Chelon labrosus*) has been reported by Cataudella et al. (1988).

Sea bass and grouper

Sea bass (*Lates calcarifer*) is a large perch found throughout the tropical and subtropical western Pacific and Indian oceans; in Asia, it is called sea bass and, in Australia, barramundi. It is an important food fish with an unusual life history that includes sex reversal from

male to female at 4–5 years of age. Sea bass spawn in coastal waters; the larvae remain in swamps and estuaries until their 2nd year, then move to inland waters until migrating back to coastal waters to spawn. Our knowledge of sea bass life history comes from Australian studies that form the basis of a management strategy for the wild fishery in that country (see references in Griffin 1988).

Because sea bass is euryhaline, it is adaptable to culture in inland earthen ponds or in coastal net pens. In Singapore, for example, production of sea bass from net pens now accounts for about half the total fish production; sea bass is also an important cultured fish in the Philippines and Thailand.

The need for large numbers of fry has resulted in the typical pattern of technology development: limited success using HCG in the mid-1970s and early 1980s, followed by repeated spawnings after injection and implantation of GnRH analogues. What is most interesting about the development of technology for induced spawning of sea bass, however, is the success of environmental manipulation, which has more than kept pace with hormonal methods.

Induced spawning using GnRH analogues

The early use of implanted or injected analogues of mammalian GnRH to induce spontaneous spawning in sea bass was reported by Harvey et al. (1985) in the Philippines and by Lim et al. (1986) in Singapore. As in all induced breeding studies with sea bass, the fish spawned spontaneously in communal tanks; the reproductive response of individual fish has been evaluated in further work in the Philippines by Almendras et al. (1988) and Garcia (1989), in which different routes of

administration — injection, pellets, or osmotic pumps — and different doses of pelleted analogue were compared. These papers point to the need for sustained release of the hormone (least stressfully achieved using cholesterol-based hormone implants) as well as to the effects of hormone overdose on egg quality: fertilization was significantly lower with GnRHa at 300 μg/kg than at the optimum dose range of 38–75 μg/kg.

Gonad maturation can also be advanced in sea bass, using implants of the male sex hormone 17α-methyltestosterone in combination with a GnRHa (Garcia 1990). This technique can potentially be used to extend the period of fry availability by several months.

Environmental manipulation and spontaneous spawning in net pens

Like milkfish, sea bass spawn spontaneously given adequate environmental stimuli, and sporadic spawnings in sea pens have been seen in the Philippines and Singapore. A successful spawning method based on controlled environmental manipulation was first worked out in Thailand by Kungvankij in 1986: this somewhat laborious procedure mimics the fish's presumed natural response to a rise in water temperature and salinity during spawning migration by manipulating the water level in concrete tanks.

A simplified procedure, in which salinity is not changed, has been developed by Lim and coworkers in Singapore and was compared to hormone injection and spontaneous spawning in net pens. Their unpublished findings show the superiority in egg quality obtained by natural spawning and argue strongly for maintaining broodstock in an undisturbed, close-to-natural state. As in all sea bass work, the lunar cycle influenced

spawning: environmental manipulation, for example, worked only at full moon, whereas fish in net pens spawned shortly after both new and full moon.

The lesson to be learned from these experiments with sea bass is the same as from recent work with milkfish: injecting or implanting spawning hormones can be bettered by providing more of the natural environmental cues that the fish needs to mature and spawn. This can be done either by labour-intensive methods such as raising and lowering water levels in tanks, or more simply by keeping broodstock in net pens and putting more effort into collecting eggs efficiently. The same principle probably holds for most cultured fish.

Grouper

Groupers belong to the same family as sea bass (Serranidae) and, like sea bass, are highly prized in southeast Asia. Popular species belong to the genus *Epinephelus* and are protogynous hermaphrodites, changing from females to males at a relatively advanced age (7 years in *E. fario*). The chronic shortage of natural fry is a great impediment to their culture in coastal waters, as is the long wait for functional males, and induced breeding studies are often done on broodstock that have been sex-reversed by feeding them methyltestosterone.

Kuo et al. (1988) report such experiments with *E. fario*. They spawned female broodstock with multiple injections of HCG, but were unable to get fertilization of the spontaneously released eggs because spermatogenesis in the sex-reversed males had not gone far enough. As an extreme case of noncoincident maturation of gametes, grouper is an obvious candidate for sperm cryopreservation.

South and Central American species

Freshwater fish culture in South and Central America is growing rapidly and, although imported species including tilapias and carps are important, there is also tremendous potential for growing indigenous species that perform well in culture and are valued by local people (Saint-Paul 1986). One important group of indigenous fishes is the characins and includes *pacu*, the closely related *tambaqui*, and the *curimbata* (*Prochilodus* spp and several species of *Brycon*) — the common names used here are Brazilian; the fish have different names in other countries.

The development of induced breeding of South and Central American species has, in a sense, been the development of hypophysation itself. The technique originated with von Ihering in the 1930s in Brazil with *Prochilodus* and has been refined over the years through the influence of the Hungarian Woynarovich. Hypophysation, as it is now practiced for characins in Brazil, is an effective procedure using standardized doses of whole, acetone-dried pituitaries from ripe common carp or *Prochilodus*, 5–6 mg/kg calculated as weight of pituitary to weight of female brood fish (dose for males is 2–3 mg/kg). Acetone-dried pituitaries are easy to prepare and store without refrigeration; matching donor and recipient weights is no longer practiced. Pituitaries are injected intraperitoneally as glycerol suspensions of whole macerated glands, with the total divided into two injections of 10% and 90% of the dose. Degree of ripeness is usually assessed using external characteristics, with position of the germinal vesicle a secondary indicator; time to ovulation after the second injection is temperature dependent.

This simple hypophysation procedure is described in Woynarovich and Horvath (1980; and 1983 in Portuguese). Although ovulation success can be as high as 80% using the technique, a reliable supply of high quality pituitaries is essential, and shortages of the glands have prompted many farmers to try HCG and GnRH analogues. HCG has not proven cost effective alone or in combination with pituitaries, and recent efforts have concentrated on GnRH analogues.

Recent results using GnRH analogues have been summarized in Carolsfeld (1989) and Carolsfeld et al. (1988). Although induced final maturation and ovulation can be achieved in characins using [D-Ala^6, Pro^9 NEt] LHRH, few data are available on fertility and subsequent larval performance. A notable feature is the need for better assessment of gonadal maturity in females: position and condition of the nucleus or germinal vesicle appear not to be as reliable indicators of ovarian maturity in characins as they are in many cultured species.

GnRH analogues are particularly potent in *tambaqui* (3–5 μg/kg as a single injection) and *pacu* (Bernardino et al. 1988). Doses of 5–10 μg/kg are also effective in some species of *Prochilodus*. These doses represent a 100-fold cost saving over HCG and a 10-fold saving over pituitaries; the reduced handling with single injections is also significant. Preliminary trials with Ovaprim-C®, a commercial "Linpe method" spawning kit, were successful but suggested that dopamine inhibition may not be important in *Pacu*.

Pinheiro et al. (1987) describe broodstock management procedures that provide ripe *tambbaqui* throughout the year in northeastern Brazil.

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Chapter 5

Assessment of maturity in breeders

Some fish mature only so far and so fast in captivity. If the conditions of captivity cannot be changed, maturation must be forced with hormones. Much of the practical information in this book can be reduced to the following: *which* hormone to use, *how much* to use, and *when* to use it. The last variable — the *when* of hormone application — is crucial.

Successful hormone-induced final maturation, ovulation, and spawning depend on accurate information about the state of the gonad. There is definitely a "right time" when hormone treatment is most effective at the lowest dose and with the fewest injections. However, culturists around the world work with different species held under different conditions, they use different hormone preparations and their final aim — strip-spawning or spontaneous spawning — varies too. Success with each species depends on understanding its reproductive performance in a given culture situation and being able to judge the reproductive state of breeders at any time.

In the face of variability in species and holding conditions, the best overall strategy for predicting readiness

is to gather baseline information on the fish in question, held and treated in conditions as standardized as possible. Where a lot of information has been published on readiness in a particular fish, culturists can at least follow existing guidelines for temperature, salinity, time in the spawning season, tank size, and handling with some confidence that these published methods will apply to them as well.

In many tropical fresh-water species, fish mature or "ripen" to a certain point but need appropriate environmental and social signals to complete maturation and spawning. Forced reproduction at this stage is best described as "induced final maturation and ovulation," and a certain minimum maturity must be reached before induction will work. Salmonids are at the other end of this spectrum, as they carry their maturation through to ovulation and then wait for cues to start spawning; in these fish, induced reproduction generally consists of speeding up the process, at the same time synchronising the reproductive responses of different individuals. Where conditions are unique or a new species is being bred, the best approach is to keep good records of state of readiness — however it was judged — so that successful methods can be repeated.

Judging readiness in females

Female readiness can be judged by methods that range from quick and easy (*external appearance*) to complex and time consuming (*egg diameter*, *size distribution*, and *morphology*); rapid and sophisticated biochemical tests may also appear in the coming decade. The best approach is to develop a practical, well-documented process that relies on several of these options.

External appearance

In most species, ripeness reflects enlarged gonads and a "loosened" follicular structure. This results in the characteristics most commonly used for selecting ripe female fish: large, soft abdomen and a swollen gonadal papilla. These subjective, shorthand descriptions of a minutely shaded range of characteristics, when made by someone with skill and experience, can give excellent results. Selection based on external characteristics is the least stressful of all methods, and most farmers evaluate fish that are still in the water. The technique is less useful for inexperienced farmers and new species, and it can miss fish that are ripe and responsive but have small gonads (low fecundity).

Once fish have been injected, it is often useful to monitor their progress if they are to be stripped. For some marine and brackish-water species, the increase in abdominal size and softness due to gonadal hydration is a good indication that the hormone is having the desired effect. In addition, some fish have distinctive behaviours, or even vocalization, that immediately precede spawning. In at least some species, a period of twitching of the dorsal musculature typically precedes ovulation by several hours, and can be used as an indicator.

Gonadal biopsy and egg analysis

Eggs are easily removed from the ovaries of many species, and their size and appearance can be excellent indicators of readiness. Three methods for removing eggs can be used.

- First, cannulating the gonoduct with fine polyethylene tubing (2–3 times the diameter of the oocytes while still passing easily through the genital

opening) and aspirating by mouth or preferably by syringe is probably the most widely used biopsy procedure. With a little practice, a person can insert tubing into an anesthetized fish (being careful to push to roughly the same spot in each fish, to reduce sampling error), and gently suck out a few dozen eggs. The basic technique works for carps, catfish, milkfish, mullet, rabbitfish, *pacu*, *tambaqui*, Asian sea bass, grouper, and many others.

- Second, puncturing the abdominal wall with a hypodermic needle and withdrawing gonadal material while moving the needle up and down within the gonad is less used but can be effective and quick, especially with less ripe individuals. In some species with fragile oviducts, abdominal puncture does less internal damage than cannulation.

- Third, incising the abdominal wall and removing larger samples of ovarian tissue is most applicable to larger fish, fish where standard biopsy procedures do not work, or when intact gonadal material is needed.

What can be learned from looking at eggs? The process of gonad maturation is gradual, and a ripe female typically has a mixture of oocytes in various stages of germinal vesicle (nuclear) migration. As the gonad matures to the point where a farmer would consider the fish to be "ripe," most of the oocytes still have centrally located nuclei, about a third have displaced (migrating) nuclei, and a few may already be mature with nuclei that are peripherally located or have already broken down. By the end of the ripe period, the proportion of mature oocytes has risen to about half. Beyond this point, oocytes of all stages may become enlarged and

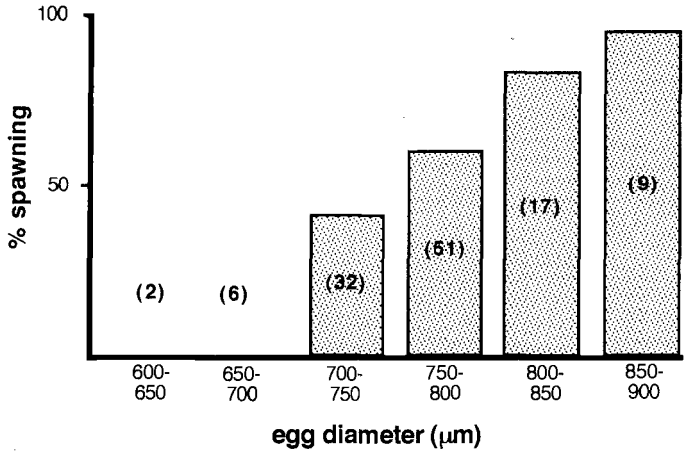

Fig. 9. Spawning success in milkfish using a single injection of GnRH analogue, showing the importance of average egg diameter. The numbers in parentheses indicate numbers of spawning attempts for each size class. (Redrawn from Lee et al. (1986).)

atretic if ovulation and spawning are not induced. Females generally produce the largest number of viable eggs if injected during the early to middle period of ripeness.

Oocyte growth, position of the nucleus, and accumulation of yolk can all be used to judge readiness. The more background information is available on egg size and morphology during development, the more accurate the methods are: for species such as milkfish and mullet that have been studied intensively in well-equipped laboratories, much baseline information exists and predictive methods are well developed (Fig. 9).

Egg diameter and size distribution

Egg diameter is easy to measure, and both *average diameter* and *size distribution* are useful indicators of readiness in many species. All one has to do is expel the

eggs into a fixative such as 5% phosphate-buffered formalin, put some in a petri dish and use an ocular micrometer or stage grid to measure a sample large enough to be statistically reliable. Egg diameters accompanying successful induced breeding have been published for many fish. Use these as a guideline and, more important, follow a standard biopsy, fixation, and measurement procedure to eliminate variability. It is especially important to make sure that the fixative does not shrink the eggs, as it will if it is hypertonic to them.

In some fish where much research has been done on ovarian development, *egg size distribution* is a valuable predictor. In milkfish, for example, response to GnRHa injection is best when average egg diameter is greater than 750 μm and there is either only one size class of eggs or when the smaller group is less than 400 μm in diameter. This is because the milkfish ovary is group synchronous (see Chapter 2) and final maturation can be induced when the ovary has a uniform clutch of large oocytes and a more variable clutch of smaller ones. Once milkfish eggs reach 750 μm, there is a critical period of about 1 week in which induced spawning with GnRHa will work. Egg size distribution is particularly useful in species where egg diameter only increases slightly after the end of vitellogenesis.

Egg morphology

Nuclear position and the number and size of yolk droplets can also be used to predict readiness, and a migrating or eccentric nucleus is easy to see and often diagnostic. Egg cytoplasm can be cleared to reveal the position of the nucleus, and counts of eggs at various stages indicate the overall maturational state. Clearing can be done with Serra's fluid (40% formalin, 40% ethanol, and 20% glacial acetic acid); clearing should be

done in a well-ventilated place so that formalin fumes are not inhaled. Acetic acid drops alone can also be used. In some species (e.g., grass carp), the nucleus is visible without clearing. Nuclear position can often be judged with the naked eye, although examination using a dissecting microscope is more accurate.

Postvitellogenic oocytes can be characterized as "central," "migrating," "peripheral," or "mature," based on the position or absence of the nucleus. The relative proportions of these categories indicate ripeness in most species. Care should be taken, however, to ensure that clearing is effective and that the nucleus is really being seen. If the acetic acid or the clearing solution being used has not been stored well sealed, it may not clear the cytoplasm entirely, leaving a central uncleared portion that can be mistaken for the nucleus. Likewise, if the clearing solution is too strong, or clearing is too prolonged, the nuclear membrane clears as well, and oocytes appear devoid of the germinal vesicle. Clearing is effective for fresh oocytes and ones that have been fixed in 1% formalin for less than 1 month. The clearing is also transient: 1–2 minutes are needed for the clearing to take place, and then the nucleus is only visible for 4–5 minutes. The same eggs can be cleared again if necessary.

Time limitations in egg sampling and analysis

Nuclei are more easily observed in some species than in others and, although the process can be done in minutes with some species, it may require more observation and note-taking than many practical operations will allow. Even measuring 50 oocytes and calculating their average diameter takes several minutes, and this delay creates practical problems in organizing a smooth induced-spawning procedure. Practice can alleviate

these problems to some extent, but better techniques are needed.

Breeding fish are often tagged and placed in small holding pens while their eggs are being measured or analyzed, then recaptured for injection or released for a few more days. In line with the overall aim of reducing handling, such delays should be minimized. One way is to keep the breeding fish lightly sedated in a small enclosure, but this requires tests with different concentrations of anesthetics.

Judging readiness in males

Males frequently produce milt in captivity and the problem of judging their readiness is not nearly as great as in females. Storing milt — for a few days or even through a breeding season — is also much easier than storing eggs (see Chapter 7). A spermiating male has sperm in the lumen of the testis and sperm duct. The next step, comparable to final maturation in the female, is "hydration" of the sperm by secretion of seminal fluid: volume of sperm increases dramatically and the sperm becomes more fluid and dilute. Captive males may remain in this condition for several months without spawning, but the testis is eventually reabsorbed if spawning does not happen.

Ripe males are usually easily distinguished by a soft abdomen and the release of milt when the abdomen is squeezed. In some species, secondary characteristics may become pronounced too. Characteristics of the semen are diagnostic within species: more free-flowing and dilute semen indicates more advanced maturity, whereas bloody semen usually means the male is spent.

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Chapter 6

Natural reproduction and environmental control

Experience of zoo keepers

Fish farmers are not immune to "tunnel vision," an affliction that causes people to think their problems are unique and that prevents them from seeing solutions — or even ideas for solutions — in the experience of others. Yet in induced reproduction of fish approaches other than the hormonal one should be tried because they have proved highly successful in other kinds of animal husbandry.

Consider zoos. Zoo keepers have been maintaining captive animals from all over the world for over a century; in fact, "zoo keeper" is an inadequate term now, because the people charged with deciding where to hold animals, what to feed them, how to keep them healthy, and especially how to breed them are likely to have advanced degrees in veterinary science, reproductive endocrinology, or animal behaviour. Since the 1960s, an entire discipline — zoo biology — has arisen out of the need to understand and modify the behaviour of captive

animals. The zoo keeper's need to breed the animals is as urgent as the fish culturist's need to breed the fish, if for different reasons. The modern zoo depends on animals propagated in captivity rather than captured from the wild, and has in fact become a sophisticated means of rescuing and maintaining endangered species.

The challenge for zoo keepers and fish culturists is really the same: to provide the conditions for spontaneous mating and reproduction. By and large, although hormonal methods of forcing reproduction in zoo animals have worked to some extent, results have been improved by providing the appropriate environment. These days, hormones used in zoos are more likely to be contraceptive!

Some of the successes in zoo-animal breeding are striking. One misconception that can be put to rest is that, in order to breed, animals need an environment very similar to their native one; a good example of animals reproducing outside their native range is the routine breeding of penguins and flamingos in the British Isles. The cues an animal needs for breeding are not necessarily the obvious ones of space and "natural" surroundings. Cheetahs, for example, as mobile an animal as one could imagine, will not breed so long as males and females are kept together, no matter how big the enclosure; if they are allowed to meet once the female is in season, however, breeding goes ahead — again, even in the British Isles.

Other examples abound, like the antelope-like gerenuk whose urine carries reproductive pheromones that get diluted out if the animal drinks too much water, or the rare Chinese alligator that breeds in captivity only when its body temperature is lowered. Equally, naturalistic surroundings are not necessarily important, as

shown by the Congo peacock, which breeds sooner in a dilapidated but undisturbed aviary than in an elaborate, park-like display setting.

There are some useful principles for fish culturists in these examples. One is that although animals breed when they are given the right conditions or cues, those conditions or cues are not always the obvious ones (such as space); they must be found out by trial and error that amounts to a thorough knowledge of the animal's life in and out of captivity. Another principle is that hard-to-breed animals often do better in "off-exhibit breeding" — large tracts of land or "breeding parks" where animals move freely and are not on display. The counterpart in fish husbandry might be broodstock-holding facilities that serve no other purpose and where disturbance — including periodic raiding for subjects for induced breeding experiments — is minimal. A third and encouraging observation is that captive breeding of exotic species can be remarkably successful. Tigers, for example, are now routinely on contraceptives at many zoos, and their offspring are a glut on the market.

Fish, of course, are not birds or mammals or even reptiles, but we should not make the mistake of ignoring the lessons learned in the husbandry of the higher animals. Large public aquaria still tend to do so, believing that veterinary science and equipment are somehow irrelevant to care of captive fish, and that fish are easily replaced from the wild. Of the more than 200 fish species now recognized as endangered, only nine are breeding in captivity — seven of these at one aquarium. Important parallels can, however, be drawn for the vital importance of diet in broodstock conditioning, the quality — not necessarily quantity — of space the animals are held in, and the need for animals to carry out courtship.

At present, with some notable exceptions, broodstock fish for aquaculture are treated the way potential breeding animals were treated in zoos 30 years ago. Capturing broodstock fish for injections of spawning hormones is the equivalent of using a "squeeze cage" to restrain a zoo animal: by the time, the animal is immobilized, it is severely stressed and the treatment may not work at all. The modern zoo keeper prefers to deliver drugs from a blowgun rather than inject them directly into a struggling animal, and fish culturists should adopt the same attitude by disturbing their valuable broodstock as little as possible. One excellent way to do this (as there is no blow-gun equivalent for fish) is to stop handling the animals at all, and to provide them instead with the conditions under which they will mature and spawn spontaneously.

Experience of tropical-fish breeders

Fish culturists have deadlines and production quotas, and persuading administrators and owners to put more effort into achieving natural reproduction, when hormone-induced reproduction may be at least partially successful, will be difficult. Nevertheless, the arguments for paying more attention to broodstock needs are powerful. The dividends paid by experimenting with holding conditions can be large, and will almost certainly include higher quality gametes. Some of the best evidence that the natural spawning method works comes from the world of tropical aquarium fish.

Tropical-fish fanciers consider breeding of any recently collected species a challenge, and the huge demand for young of all popular tropical aquarium fishes has resulted in large and specialized fish-breeding industries in Hong Kong, Singapore, and the

USA (Florida). Tropical aquarium fish bred in captivity cover as wide a range of species as are farmed for food and breeding technology is well documented in a number of standard works that fish culturists could profit from (e.g., Axelrod 1987).

Most of the breeding is by natural means, and successes are legion. The example of the "Dragon Fish" or Asian *arowana* is really no different from the milkfish or sea bass that spawned when they were placed in undisturbed net pens. In the case of the *arowana*, young fish are desired for display, can no longer be captured legally, and command a very high price, but the strategy that worked for captive breeding in Singapore was simply to provide undisturbed space, water of the correct hardness, and high quality diet.

Much has been written on breeding tropical aquarium fish and, although most of it concerns freshwater species and is probably transferable to marine cultured fish only in intent rather than in detail, there is still an enormous amount of practical information on fish from groups important in aquaculture: characoids, cyprinids, and catfishes in particular have been bred with great success following well-established general guidelines.

Principles of tropical-fish breeding

In describing successful breeding of new species, aquarists return time and again to the vital importance of *diet*, *water quality*, and *breeding site*. The factors interact, of course, and vary in importance with the species, but they determine success or failure. A good example is the zebrafish, a river-dwelling characoid from India that is one of the most popular aquarium fishes and one of the easiest to breed — as long as certain rules are followed. Zebrafish pairs breed prolifically

as long as they have first been kept apart from each other and conditioned with high-quality food; they are often used as a laboratory animal to produce embryos for study, and a pair will spawn in a completely bare container as long as the water is the right temperature and hardness and they have been well fed. The important point is that spawning in a *communal* tank, even one well planted with spawning substrate, will be feeble or nonexistent if diet and water are inadequate. Broodstock on a farm are really no different.

Diet in broodstock conditioning

Broodstock conditioning is vital in successful breeding, and aquarists devote much time and energy to offering the right diet to their breeding fish. The right diet for conditioning, which is just another term for promoting gonad maturation, is almost always live food or high quality freeze-dried food, and sexes are almost always separated for conditioning.

Most of the time, trying to breed fish in a communal tank does not work. However, once separated and fed well with high quality food, the females develop distended bellies and the males become more deeply coloured. It is always at this point that the aquarist puts a pair together for final maturation, courtship, and spawning.

The lesson for fish culturists is clear, and much more effort should be put into improving broodstock diet during conditioning. Obtaining high-quality food, even for a limited conditioning period that may last only a few weeks, is an obvious problem for many culturists, but if they are successful in this single most crucial area of broodstock management, the dividends will be great.

Crucial components of a "high-quality" diet for

reproduction generally are high protein level (with appropriate amino acid composition) and adequate amounts of essential fatty acids, minerals, and vitamins; exact requirements differ between species. Literature is also sparse on this topic, but the farmer should learn as much as possible about the specific amino acid, vitamin, and fatty acid needs of the species in question and be careful to provide these during maturation.

Water quality

Successful aquarium-fish breeders are scrupulous about finding and maintaining the right levels of pH, temperature, and dissolved solids ("hardness") for their breeding fish. Salinity is an important variable for marine species. Preferences vary widely and usually reflect the natural situation — another reason for knowing the wild habits of one's fish. Lake fish prefer hard, alkaline water, whereas stream fish spawn more readily in soft, acid water; more specific requirements are best learned by studying the animal in the wild. Fish culturists trying to condition broodstock should, as a matter of course, monitor and adjust pH, hardness, and salinity to the best of their practical ability and consider these in designing the holding facilities.

Breeding site

Species vary in their physical requirements for spawning: some require a hiding place and others need plants on which to deposit their eggs; some scatter eggs in open water without site preparation or parental care of the embryos and larvae. As a general rule, the conditions for *maturation* and *spawning* are different, so that a fish may develop ripe gonads in relatively bare surroundings but be unable to go through courtship

behaviour and spawning until the right physical cues are provided.

Those culturing tropical fish go to great lengths to find out what their "ripe" adults need in the way of spawning substrate and, if fish farmers see enough benefit in natural spawning, they will have to determine these for their own fish through a combination of trial-and-error and learning about the behaviour of their fish in nature.

Spontaneous reproduction in cultured fish

Fish *spawn* when environmental conditions ensure maximum fertilization and larval survival. However, the gonads often *mature* under a different set of conditions and this makes manipulating the environment to control the entire process a challenge. When we think about ways of manipulating the environment to induce maturation and spawning, it is useful to review the response of the endocrine system to the environment.

As we saw in Chapter 2, gonadal growth (oogenesis and vitellogenesis in females; spermatogenesis in males) is influenced by fairly long-term predictive "cues," the most common being daylength and temperature. Final maturation, on the other hand, occurs in response to more specific, short-term or synchronizing cues such as the presence of aquatic vegetation or pheromones from a mate, and leads to actual release of gametes (spawning).

In reality, it is difficult to assign specific cues to each stage of reproduction and much more needs to be known about predictive cues before they can be used reliably to

spawn fish in captivity. For some fishes, the same cues will affect both gonad growth and final maturation and spawning, whereas in other species additional synchronizing cues will be needed. This important subject has been reviewed by Lam and Munro (1987).

Advantages of natural maturation and spawning

What are the advantages of natural maturation and spawning? A partial answer is that, by providing the conditions for natural breeding, the culturist no longer has to worry about most of the topics in this book — things like stress, anesthesia, fertilization technique, choice of hormone, and gonadal biopsy become irrelevant. It is also true that, by and large, hormonal methods do not work well for all species and the effect of repeated handling is to further reduce the effectiveness of a treatment. Natural *spawning* alone has advantages over stripping in fishes where egg quality deteriorates rapidly after ovulation.

Although control of the environment obviously has its own set of problems, the benefits of natural reproduction extend beyond simply avoiding the complications of hormone technology. At the time of writing this book, one of the most active topics in seed production and larval rearing is the importance of broodstock management. More than ever before, researchers and farmers are finding that broodstock diet strongly affects not only fecundity but also the biochemical makeup of eggs and sperm as well as the growth rate and survival of larvae (Foscarini 1988).

The term "egg quality" is frequently used and, although not a very accurate one, it conveys the idea of evaluating reproductive output in ways other than by

numbers alone. A striking example of how invisible differences in gamete quality can affect later performance is provided by the cryopreservation of eggs and sperm: gametes obtained late in the season or from poorly fed broodstock look healthy, but cannot withstand the artificial challenge of freezing (Chapter 7).

Examples of natural spawning

Some important recent examples of natural spawning in "hard-to-spawn" species have already been discussed in the sections on milkfish and sea bass (Chapter 4). More and more such cases are being described. There are reports of natural tank spawning in Atlantic halibut and cod and the fast-swimming, warm-water fish *mahi-mahi*, all species that have been considered difficult to reproduce by hormonal means. However, it is worth noting that these fish were all spawned at well-equipped laboratories with good facilities for egg collection, and individual culturists who wish to attempt natural spawning as an alternative to stripping of hormone-treated fish will have to balance the possible improvement in egg quality against the possible loss of eggs.

The effort that can be put into developing a system for natural maturation and spawning depends not only on the physical resources available but also on the size of farms and the way individual farmers want to operate. For example, it may be entirely feasible to control broodstock diet and environment in a well-equipped hatchery, but local farmers may not want to deal with a central seed supply, preferring to produce their own seed by hypophysation. Thus, any discussion of environmental control, natural maturation, and natural spawning is necessarily general.

Combined approaches can work well, such as the use

of water sprays and currents in breeding tanks for Indian carps; in this case, hypophysized breeders were spawned naturally outside the normal breeding range and season by lowering water temperature and increasing dissolved oxygen (Dwivedi and Reddy 1986). Providing environmental cues to hormone-injected fish might be called a "seminatural" approach, and should be considered where timing of stripping is critical.

Unfortunately for tropical-fish culturists, most of the work on cues for gonad growth, final maturation, and spawning has been done on temperate species, particularly salmonids. Not surprisingly, the most important influence on gonad growth in these fishes is changing daylength. Daylength varies much less in the tropics than in temperate climates; for many warm-water species, the most important environmental influences on gonad growth are temperature and the annual cycle of rainfall. Flooding produces many measurable changes in water quality including a reduction in dissolved solids and often temperature.

Fish culturists can use this information by mimicking changes in water quality and temperature, an interesting example being the use of overhead jets of water to increase dissolved oxygen and to cool tanks containing breeders of Indian carps (Dwivedi and Reddy 1986). Another example of the importance of temperature for gonadal growth comes from controlled studies on the Chinese catfish in Hawaii; photoperiod was found to influence gonad maturation, but to a much lesser extent than ambient temperature (Young et al. 1989).

Fortunately for tropical-fish culturists, water temperature, dissolved solids, oxygenation, and even current flow are more easily controlled than photoperiod. Also, the potential impact of such straightforward manipula-

tions as raising and lowering water levels, creating currents, and providing spawning sites is great. Another example of the benefits of environmental manipulation comes from the whitefish in South Africa, where natural spawning in both hypophysized and untreated fish was stimulated by providing an enriched broodstock diet at low stocking densities and spawning substrate at the inflow of the spawning pond, and simulating flood conditions by moving fish from a stagnant conditioning pond to a previously dry pond in the process of filling (Bok and Immelman 1989).

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Chapter 7

Preservation of sperm

Most of this book deals with reproduction in female fish. We have already seen that males often produce milt in captivity, although the milt may be viscous and in low volume. Many researchers and fish culturists have realized that because males are easier to deal with in culture — even if they have to be injected with a hormone to spermiate, the response is predictable — the ideal situation would be to have sperm "on the shelf," ready to use when the more difficult-to-induce females have ovulated. Also, when males mature before females, all the milt in the world is useless if it cannot be stored.

Convenience alone is the major reason so many fish culturists have tried to store milt. There are other important reasons, all based on the argument that has made artificial insemination such a vital tool in livestock husbandry — spermatozoa from individuals with desirable genetic makeup can be used in selective breeding. Sperm banking is equally applicable to culture in enclosures and wild-release or "ocean ranching" forms of mariculture, and has enormous potential significance for aquaculture as genetic improvement programs gather momentum.

A *gene bank* of frozen fish sperm is an excellent,

cost-effective tool for conserving biodiversity in cultured species of fish. Genetic "bottlenecks" created when too few broodstock are relied on for larval production can be eliminated by incorporating frozen sperm from more distantly related males into the breeding program, thereby significantly increasing effective broodstock population size. In many cases, these more distantly related males are also geographically distant from the culture site — perhaps even in the wild — and transporting frozen sperm is the only option for making their genetic material available to breeders. Sperm banks can also be used to preserve the genetic diversity of species under environmental pressure.

Spermatozoa can be *chilled* for a short period (up to several weeks) in a refrigerator at about 5°C, or *frozen* and stored in liquid nitrogen (–196°C) for years. Chilling and freezing are completely different processes and are discussed separately.

Short-term or chilled storage

Milt can be stored at about 5°C for periods ranging from a few days to several weeks. Until more is known about the control and prolongation of sperm motility, the best strategy is to store milt undiluted. Adding broad-spectrum antibiotics can prolong storage, but the right concentration and method of addition must be found through trial and error. Cryoprotectants — chemicals used to prevent ice-crystal damage (see later) — have no function at temperatures above freezing and are not needed. Containers must allow for gas exchange, and a period of standing at room temperature, after removal from the refrigerator but before fertilization, has been found to improve fertility.

Storage of milt for as little as a few hours can be very useful in induced breeding, allowing culturists to concentrate on stripping females or to transport milt over short distances. Some references on short-term storage of sperm are provided at the end of this chapter.

Cryopreservation

The spermatozoa of fish are simpler than mammalian spermatozoa — they lack the acrosome for digestion of the egg membrane — and are easily frozen to the temperature of liquid nitrogen and stored indefinitely. There are reports of more or less successful sperm cryopreservation in most cultured species, with fertilization and hatching levels as good as with fresh sperm. There are no convincing reports of successful freezing of a fish egg or embryo and, if techniques to do this are eventually worked out, it will be in specially equipped cryobiology laboratories.

If fish sperm can be "easily frozen," why are published results so often unsatisfactory? The answer lies in the science of *cryobiology* — the study of the effects of low temperatures on living systems. Cryobiology is not like endocrinology, where basic principles are well understood and further refinements make practical techniques — such as administering GnRH — possible. The effects of low temperature on cells are *not* well understood, and most theories in cryobiology have evolved to explain trial-and-error results of freezing various biological systems. The inevitable result of this lack of a solid foundation is that people trying to freeze a new cell type, such as the spermatozoa of a particular cultured fish, can only copy methods that worked for other animals. Unfortunately, all cells respond differently to freezing.

Nevertheless, freezing fish sperm is not inherently difficult, and persistence is paying off as successful techniques are reported for one fish after another. In an effort to speed up the process, the following discussion covers the major points where confusion can wreck a cryopreservation attempt. The theory is straightforward: milt is *diluted* with a diluent containing one or more *cryoprotectants* that reduce ice-crystal damage, *cooled* in various containers on dry ice or in liquid nitrogen vapour, *stored* (usually immersed in liquid nitrogen), then rapidly *thawed* for addition to eggs.

Importance of gamete quality

Culturists know that gamete quality varies; the eggs of some females, and the milt of some males, are more fertile than others. Sperm frozen early in the spermiation period is usually more fertile than sperm frozen at the end of spawning. This may have something to do with changes in the composition of the sperm-cell membrane during the breeding period (see discussion of damage to sperm cells, later). Egg quality can also strongly affect the outcome of sperm-freezing trials, and must be standardized by fertilizing eggs from the same female whenever sperm treatments are compared.

Sperm quality also deteriorates after removal from the fish, but there is no clear trend that might recommend immediate freezing over chilling the milt for various lengths of time before freezing. The safest course, as always when no biological explanations exist, is to standardize the delay between extraction and freezing, and to keep it to a few hours at most.

Diluents and cryoprotectants

If untreated milt is cooled to the temperature of dry ice or liquid nitrogen, two forms of fatal damage occur:

first, ice forms inside the cell and, second, the dehydration that takes place during cooling results in membrane damage as the salt concentration outside the cell increases (see Fig. 10). Both effects can be reduced or eliminated by adding *cryoprotectants* to the milt.

Cryoprotectants are added by mixing them in a carrier called a *diluent* (sometimes called an *extender* because it has the added advantage of reducing sperm-cell density to a level convenient for artificial fertilization). A tremendous amount of effort has gone into developing complex diluents that mimic the composition of seminal plasma, but really the only requirement of a diluent is that it carry the cryoprotectants to the sperm cells and that sperm cells are not activated in it. In salmonid fishes, for example, excellent results are now being obtained by adding cryoprotectants to the very simple carrier of 5% (300 m*M*) glucose in distilled water. Usually, the cryoprotectants themselves inhibit motility; if not, increasing the osmotic pressure of the diluent by adding sodium chloride (0.9% final concentration) is effective. Preventing sperm activation in the diluent is essential because, as we have seen, sperm cells swim for only a short period, and we want them to swim when they are in contact with eggs, not while they are being readied for freezing.

Cryoprotectants are *intracellular* (penetrating) or *extracellular* (nonpenetrating). Intracellular cryoprotectants are more important and include (in rough order of frequency of use) *dimethyl sulfoxide* (*DMSO*), *glycerol*, *propylene glycol* (*propanediol*), and *methanol*. Intracellular cryoprotectants work best at final concentrations between 5 and 10% volume/volume (the term "final concentration" means *after* dilution of the milt and the concentration of cryoprotectant in the diluent itself will be higher). DMSO at a final

concentration of 7.5% is a good all-round cryoprotectant for many cell types; glycerol has worked well in some fishes; methanol works well in some fishes and not in others. Intracellular cryoprotectants should be reagent grade but must never be added full strength directly to milt, because the heat of solution may kill the sperm cells.

Extracellular cryoprotectants often elevate fertility beyond what can be achieved using an intracellular cryoprotectant alone. They act by coating membranes and preventing salt damage. Two cheap materials work well for fish spermatozoa: powdered milk at 10–15% final concentration and egg yolk at 15–20% final concentration. Milk causes fewer problems with agglutination (spermatozoa sticking together) than egg yolk in some species.

Adding diluent to milt

When milt and diluent are mixed, intracellular cryoprotectants rapidly penetrate the sperm cells, and extracellular cryoprotectants coat the outside of the cell. There is no need to let the mixture stand; in fact, any potential cryoprotectant toxicity is minimized by cooling at once. Mixing can be done at ambient temperature. The ratio of diluent to milt determines the number of sperm cells in a given volume, and can be adjusted to a density that works well for artificial fertilization. *The final concentration of cryoprotectant is what is important, not the diluent-to-milt ratio.* For example, one might dilute milt 1:1 with a diluent containing 15% DMSO, i.e., twice the desired final concentration.

When thawed sperm is added to eggs with a little water, intracellular cryoprotectants are diluted out to levels that are not toxic to eggs or sperm.

Events during cooling

When protected sperm cells are cooled, the surrounding medium freezes at a few degrees below 0°C. As cooling continues, water leaves the sperm cells to balance the free water "lost" when the medium froze (Fig. 10). If cooling is not too fast, the cells dehydrate as the temperature falls, and ice never forms inside the cell. By about –50°C, the cells are shrunken, the danger of ice forming inside the cells is past, and cooling can be much faster. In practice, sperm should be cooled slowly to between –50°C and –80°C (the temperature of dry ice), then plunged into liquid nitrogen, which cools it almost instantly to –196°C.

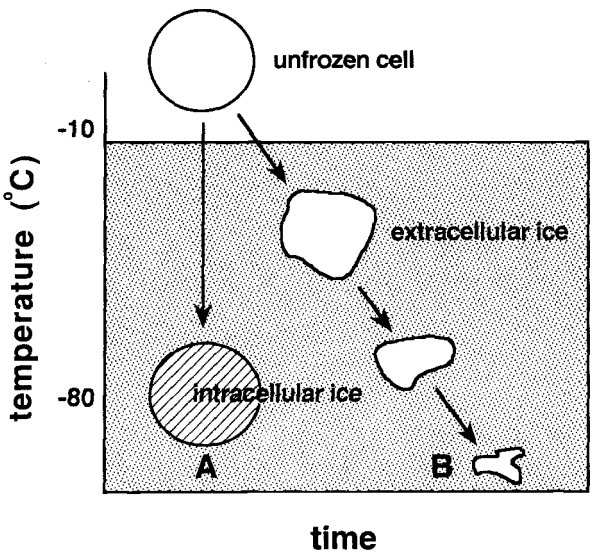

Fig. 10. Cell shrinkage correlates with cell survival in cryopreservation. A — Rapidly cooled cells do not have time to shrink; instead, they form intracellular ice and are dead on thawing. B — Cells cooled more slowly lose water, shrink, and survive because only extracellular ice is formed. For most cell types, cryoprotectants must be added to increase survival of frozen–thawed cells. (After Farrant (1980).)

When frozen sperm is warmed, ice formed outside the cells thaws and water reenters the cells. *In most cases, a proportion of the sperm cells begin to swim spontaneously, even though they were not motile before freezing.* This common effect means that the cell membrane has been altered by freezing and thawing. The fertilizing ability of the thawed sperm is not affected but, because the period of motility is short, thawed milt must be added to eggs without delay.

Rates of cooling and thawing

Fish spermatozoa should be cooled at between about 10 and 50 C° per minute, and thawed as rapidly as possible. Cooling and thawing rates depend on the container used for freezing, and one container that gives consistent results is the *plastic straw* used to freeze cattle semen (0.25 or 0.5 mL). Pellet or droplet freezing in depressions on dry ice also works but is hard to standardize and, of course, requires dry ice. Straws, on the other hand, can be laid on a hand-made wire rack held over liquid nitrogen in an insulating container. As long as the container is allowed to equilibrate, the distance above the liquid surface determines the cooling rate, and rates are repeatable. A thermocouple thermometer measuring as low as –60°C is essential for determining the configuration to use (the thermocouple tip should be placed inside one of the straws).

Rapid warming of straws is easy: gently agitate the straw in hot (50–70°C) water. If any water contacts the thawed sperm, the cells will be activated, so some care is needed. Straws should be warmed *only until the ice starts to melt* and the contents can be expelled onto the eggs.

Fertilization using frozen–thawed sperm

Spermatozoa stored in liquid nitrogen remain fertile indefinitely, and should be thawed only when fresh eggs are waiting to be fertilized. The amount of frozen sperm to use should be based on whatever is commonly done with fresh milt. Up to a point, reduced fertility can be made up for by using more milt. The process of fertilization is the same as with fresh milt, with the warning that thawed milt is probably already partially motile and must be added to eggs without delay.

Judging the success of cryopreservation

In theory, by following the guidelines above, researchers can develop a successful sperm-freezing method relatively rapidly. The fact that this does not always happen reflects a major stumbling block: if the reason for developing the technique is to overcome the problem of not having ripe eggs and sperm available at the same time, how can one test the fertility of stored sperm? Cooling experiments take only an hour or so, but it is hard to maintain interest in an experiment if the only way to test the results is to wait weeks or months until eggs are available. Typically, liquid nitrogen is obtained for a short period only, and maximum use must be made of it.

A great deal of contradictory material has been written about the relationship between sperm *motility* and *fertility*, and there are certainly cases where eggs appear to have been fertilized by nonmotile sperm. In practical fish-farming terms, however, vigorous motility goes along with fertility, and the basis for a cryopreservation method *can* be worked out using motility alone as an indicator of success.

To make confident predictions based on motility, however, researchers must do two things: they must be very

familiar with the osmotic behaviour of the sperm cells they are working with — what sort of solutions activate the cells and for how long — and they must be able to assess motility rapidly and reliably. Many research projects fail because motility has not been monitored at every step: before dilution, after dilution, and immediately after thawing. Because motility can last as little as 30 seconds, researchers must always check it the same way: by putting a tiny drop of fluid on a microscope slide and examining it *immediately* under a prefocussed objective lens, using phase contrast if possible. Cover slips inhibit motility by limiting oxygen supply, and they also take too long to apply.

By following these rules, even very short-lived motility can be detected and used as a criterion for experimenting with cryoprotectants, diluents, and cooling and warming rates. Testing frozen–thawed sperm with fresh eggs should be done only when satisfactory motility has been achieved. In studies where fertilization, development, and larval performance have been measured, cryopreserved sperm has proven as effective as fresh sperm, with no increase in abnormalities.

Future developments

The use of cryopreserved sperm in aquaculture will increase dramatically as we learn more about the control of sperm motility. At present, we know motility is inhibited by high concentrations of potassium in the testis and is initiated by potassium dilution. Studies on sperm physiology are now beginning to show us how to prolong and even to reactivate motility after sperm have stopped swimming, and fish farmers can look forward to simple methods that allow freezing milt when it is available and using it when it is needed.

Another trend that may revolutionize gamete preservation in aquaculture is the new technique of *ultrarapid cooling*, now beginning to replace many of the conventional slow-cooling methods used to preserve mammalian gametes and embryos.

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Chapter 8

Marking and tagging broodstock

Studies in induced breeding quickly become unproductive if records are not kept, and records are impossible to keep if individual broodstock cannot be told apart. Marking or tagging of broodstock is imperative when fish are being sorted and selected, when individuals are injected more than once, and when different treatments are compared. Long-term identification of individuals is vital in programs of genetic improvement. Fortunately, many options are available for identifying broodstock (Fig. 11), from simple methods such as threading nylon tape through the back musculature to high-technology electronic methods such as PIT (passive inductance transponder) tagging. The following discussion shows how the different methods work (and why they may fail), and then describes procedures and tags that can be adapted to many fish-culture situations.

Fish can be *marked* (by dye injection, branding, tattooing, spray painting, fin clipping, or oxytetracycline and microtaggant injection), *tagged* with external mechanical tags (dart and anchor tags, streamers, spaghetti and loop tags, clips, or disks), *implanted* with sophisticated internal tags (PIT tags, X-ray microtags,

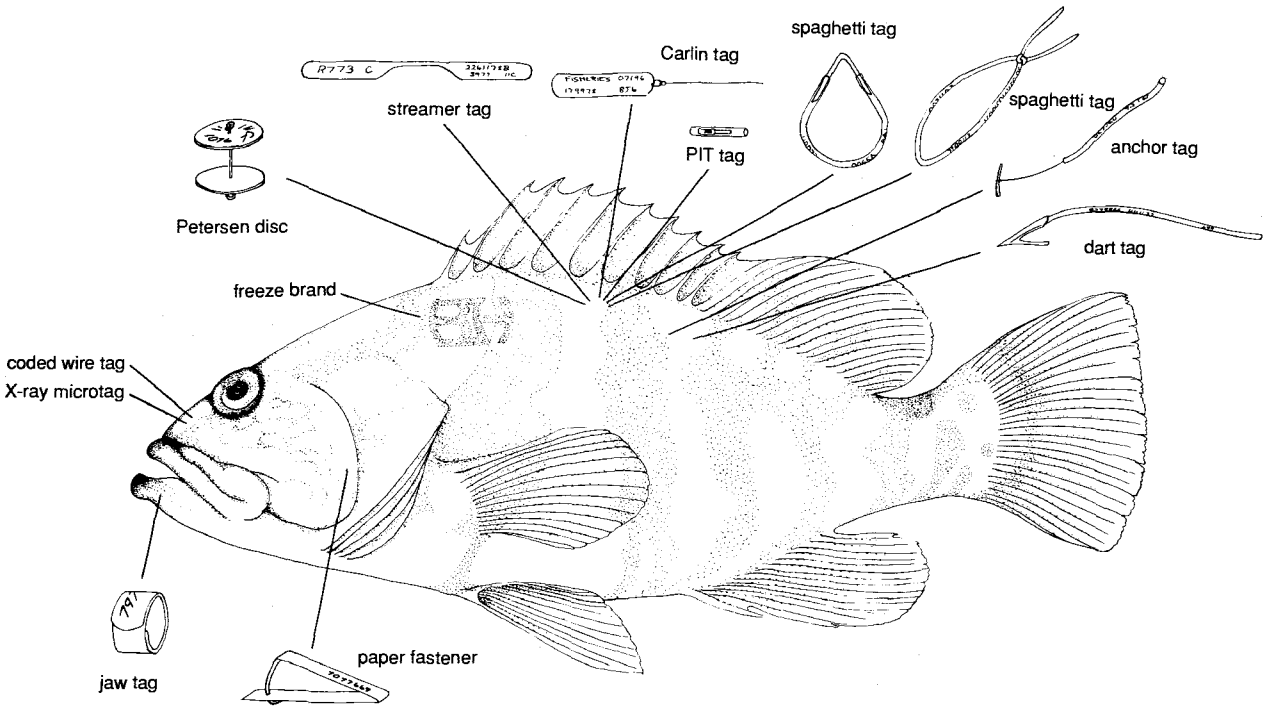

Fig. 11. A selection of fish tags and marks.

or coded wire tags) or *identified* by characteristic biochemical "fingerprints" (blood groups, meristic characteristics, scale circuli, DNA and RNA composition, or electrophoretic properties of enzymes). Requirements for identifying broodstock rule out many techniques suitable only for batch identification of small fish or requiring costly facilities, and for our purposes the list can be narrowed down to *branding* and *fin clipping*, *mechanical tags* (dart and anchor tags; disks and clips; or streamers, spaghetti, and loops), and *PIT tags*.

Tags are made by several manufacturers, who also supply associated equipment such as tagging guns. Mechanical tags in particular can suffer from separation of external and internal portions, illegibility and insufficient accompanying records, and users should inquire to avoid buying a tag with a well-known problem.

Marks

The best external marks are made by *fin clipping*, *opercular punching*, and *cold branding*. All these methods suffer from the limited amount of information that can be contained in each mark; however, individuals can be identified within limits set by the number of available locations for marks. A lesser drawback is that, when fish grow, external marks or brands are expanded or obscured; with adult broodstock, however, growth during the course of a study is probably negligible.

Fin clipping and opercular punching

Complete removal of the adipose fin is a tried-and-true method but is good only for batch marking or to alert to the presence of an internal tag. Removal of any

of the other fins, unless done at point of attachment to bone, is followed by more or less regeneration (depending on the species and how close to the body the cut was made) and affects swimming; nevertheless, irregularities at the site of regeneration have some use as identifying marks.

Partial removal of a fin is useful for studies a few weeks long at best, and holes or nicks are completely repaired within days. Fish with protruding dorsal spines can be marked using a binary system, although strong clippers are needed to cut through the bone. When pectoral fins are excluded as needed for swimming, there are *only 10 possible combinations of two-fin marks*.

Holes punched in opercula (using a paper or leather punch or modified pliers) are suitable for short-term studies only, and work best in species with bony opercula. With larger fish, the larger operculum allows a reasonable number of codes to be worked out.

Cold branding

Cold or "freeze" branding uses a branding tool cooled in liquid nitrogen and held against the dorsal integument for about 2 seconds. Branding tools are usually hand made of copper, which is easily bent and has a high thermal capacity. Brands remain legible for two reasons: there is a *primary effect* in which dark-coloured melanin-containing cells invade the branded area, and a *secondary effect* in which regenerating scales in the branded area are smaller and deformed. This secondary effect can often be seen long after the primary darkening effect has faded, particularly if the fish is viewed at an angle.

The great advantage of cold branding is that it is

noninvasive and does not affect behaviour; the main disadvantages are fading and the lack of information that can be carried on a brand. Number codes are impractical because they require numbered brands and become illegible as fish grow. In practice, the symbols used are simple shapes that are repeated, placed in different locations or combined with other marks such as fin clips. A drawback (shared with fin-clipping) is the tendency to devise over-complicated code schemes that end up by confusing everyone. Individual identification is only possible for a limited number of fish.

Success in cold branding depends greatly on scale size and temperature of the branding tool. The small scales of juvenile fish generally make them much easier to brand than adults. Fish with large, thick scales should be branded in areas where scales are reduced or absent. The branding tool (with an insulated handle) should be made of copper or aluminum and should be kept in liquid nitrogen long enough to become truly cold. It should only be removed from the nitrogen briefly for the actual branding.

Cold brands can stay recognizable for more than a year, depending on the species and the age at branding. A good way to keep brands legible is to rebrand periodically (say every 6 months); in many studies, fish are handled at least this often, and branding is simple and rapid. With rebranding and limited numbers of fish to be identified, and assuming a supply of liquid nitrogen is available, cold branding is a simple, inexpensive, and effective marking technique.

Chemical branding with silver nitrate mixed in petroleum jelly and applied to an area of the fish that has been lightly scored to remove mucous also works with some fish and removes the need for liquid nitrogen.

External mechanical tags

The great advantage of mechanical tags is their ability to bear enough coded information for large numbers of individual fish to be recognized. Most of the commercially available tags fall into this category, and prices per tag are low. Once the principles of tag retention are understood, particularly the importance of fish size, there is much scope for individual design using local materials, and many successful tagging methods are tailor-made for a particular fish and study; in fact, the diversity of fish sizes and shapes often demands this.

Secure attachment and minimal abrasion are the most important requirements in a mechanical tag. The main disadvantage of mechanical tags is the need to attach them through muscle or bone, which can result in infections that are made worse by abrasion and drag.

Anchor tags and dart tags

Anchor and dart tags are currently the most widely used external tags and are available in several sizes and from several manufacturers. *Both depend on proper implantation and will quickly be lost if applied incorrectly.*

A *dart tag* has a V-shaped plastic head molded or glued to a tubular plastic tail bearing a unique inscription or code. *Anchor tags* differ mainly in the shape of the head — they are T-shaped — and resemble the tags used to attach prices to clothing. Dart tags are inserted using a hand-held hollow needle; for anchor tags, insertion is similar but automated using a tagging gun that dispenses tags from a magazine.

Both types of tag function properly only if the barb or T-bar locks securely behind the bones supporting the

dorsal fins. Neither holds well in muscle, although manufacturers are developing modified dart tags intended to work this way. This requirement for penetration and locking between bones means that the length of tag and the length of the needle on the tagging gun must be selected with the size of the fish in mind.

As a general rule, anchor tags are more successfully used with smaller fish, because the slender T-bar can pass easily between bones, and because they can be applied with a smaller needle. However, the only reliable way to choose the proper type and size is to dissect a fish after implanting a number of trial tags. Implanting too large a dart tag will break bones, while too small an anchor tag will simply slip back out between them.

Anchor and dart tags cause wounds that can become infected. The best way to avoid such complications (as with all invasive tagging methods) is to make sure handling stress is minimized through the use of anesthetics and adequate recovery facilities. Topical antibiotics can be used to control infection.

Disks and clips

Tags come in a wide variety that can be wired or pinched onto various parts of fish. The oldest is the *Petersen disk*, which consists simply of two plastic disks held to the sides of the fish by a metal pin passing through the flesh beneath the dorsal fin. Problems with chronic lesions caused by friction can often be minimized by smoothing the underside of the disk or by custom-fabricating disks with holes for two attachment pins. The principle of the Petersen disk can be applied easily to any number of homemade tags.

Dangler tags are small plastic plates wired to the fish; the most commonly used is the *Carlin tag*. This tag

must be applied using a pair of hypodermic needles, and needs time and dexterity. Dangler tags can easily be snagged in nets.

Jaw tags and opercular tags are metal rings resembling bird bands and can work as long as they are not large enough to interfere with feeding. They can be obtained, sequentially numbered, from manufacturers of general fastening equipment. Enterprising culturists will even find that small paper fasteners can be crimped onto opercula and retained for several months; the fasteners should be painted to prevent corrosion.

Spaghetti tags, streamer tags, and loop tags

A *spaghetti tag* is a length of vinyl tubing bearing a legend or code and threaded through the dorsal musculature. A long spaghetti tag is knotted and the ends left to trail in the water; the ends of shorter spaghetti tags can be held together with special inserts so that the tag forms a circle. *Lock-on*, *loop*, or *cinch-up tags* are variants available from major manufacturers. Spaghetti tags are applied using a hollow needle and are highly visible; they are excellent for large fish in a short-term study. Loop tags take more time to attach but are more secure and less likely to be bitten off by other fish.

Another kind of tag that is threaded through the dorsal musculature is the *streamer tag* — essentially a length of tape with a narrow middle section. These can be purchased in various sizes from fish-tag manufacturers, complete with needles for insertion. They can also be hand-made cheaply from vinyl surveyors' or "flagging" tape upon which numbers are written with indelible pen. The tag is threaded onto a needle and pulled through the fish until the narrow part is in the dorsal

musculature (Blumer 1984). This method has not been widely reported, but it is simple and promising for short-term use in many species.

Internal tags

PIT tags

A PIT (passive inductive transponder) tag is about the size of a grain of rice and contains a microprocessor chip and antenna. Each tag is individually coded. When the tag is excited by a detector, it transmits its unique code to a reading device that automatically decodes, displays, and stores it. PIT tags are implanted intramuscularly or intraperitoneally using a hollow needle, and have been widely used for a number of fish and wildlife applications. For small-scale fisheries experiments, the code is detected and displayed using a hand-held reader.

The advantages of PIT tags are small size and high retention, lack of interference with normal behaviour, and virtually limitless individual codes. They are more expensive than mechanical tags, but the value of the fish and whatever treatments it has received may make the extra investment worthwhile. Also, the tags can be recovered and used again. The expense may also be justified in situations where several groups can share the equipment; however, the availability of technical support for the equipment should be confirmed.

Visible implant tags

Visible implant (VI) tags are a recent addition to internal tagging technology, and were developed to provide an externally visible mark without the negative effects of conventional external tags. The VI tag is injected into transparent tissue, usually near the eye,

and carries a code of three alphanumerics. Field tests to date indicate high tag retention, and the simplicity of the application procedure suggests that the technique will find widespread approval with fish culturists.

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Appendix

Common and scientific names of fishes

Common name	Scientific name
Asian sea bass: Barramundi	*Lates calcarifer*
Sea bass	*Dicentrarchus labrax*
Bream	*Parabramis pekinensis*
Bighead carp	*Aristichthys nobilis*
Black carp	*Mylopharyngodon piceus*
Common carp	*Cyprinus carpio*
Grass carp	*Ctenopharyngodon idellus*
Catla (Indian carp)	*Catla catla*
Mrigal (Indian carp)	*Cirrhinus mrigal*
Mud carp	*Cirrhina molitorella*
Rohu (Indian carp)	*Labeo rohita*
Silver carp	*Hypophthalmichthys molitrix*
Thai carp	*Puntius gonionotus*
African catfish	*Clarias gariepinus*

Channel catfish	*Ictalurus punctatus*
Chinese catfish	*Clarias fuscus*
River catfishes	*Pangasius* spp (*P. sutchi* and *P. pangasius*)
Walking catfish	*Clarias batrachus*
Thai catfish	*Clarias macrocephalus*
Curimbata	Prochilodus spp
Dogfish	*Squalus acanthias*
Goldfish	*Carassius auratus*
Groupers	*Epinephelus* spp (*E. fario*)
Loach	*Misgurnus anguillicaudatus*
Milkfish	*Chanos chanos*
Grey or striped mullet	*Mugil cephalus*
Mediterranean mullet	*Chelon labrosus*
Other mullet species	*Mugil liza*
Pacu	*Piaractus mesopotamicus*
Rabbitfishes	*Siganus* spp
Ratfish	*Hydolaguc colliei*
Roach	*Rutilus rutilus lacustris*
Sablefish	*Anoplopoma fimbria*
Salmon	*Oncorhyncus keta*
Sturgeon	*Acipenser transmontanus*
Ayu; sweetfish	*Plecoglossus altivelis*
Tambaqui	*Colossoma macropomun*
Tench	*Tinca tinca*
Whitefish	*Barbus andrewi*
Zebrafish	*Brachydanio rerio*

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Induced Breeding in Tropical Fish Culture is more than just a revision of IDRC's 1979 publication *The Theory and Practice of Induced Breeding in Fish* by Brian Harvey and W.S. Hoar (IDRC-TS21e). Where the first version was a more or less academic review of applied fish endocrinology, this one avoids bibliographic citations in the text except where specific techniques and research results are mentioned. Keeping citations out of the text makes the work more readable, but where technical details are essential, the reader is given all the information needed to consult the original work. A background bibliography is also provided at the end of each chapter.

This book is aimed at technicians and scientists interested either in entering the field of induced breeding or in learning about the latest developments.

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