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REPRODUCTION AND CONTROL OF OVULATION, SPERMIATION AND SPAWNING IN CULTURED FISH

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1. INTRODUCTION

Fish are the largest phylum of living vertebrates, with around 30,000 fish species out of approximately 50,000 vertebrate species (www.fishbase.com) [1]. Fishes inhabit almost every aquatic environment on the planet, presenting an enormous variation in temperature, salinity, oxygen, and other chemical and physical water properties. These environments have exerted evolutionary pressures that have resulted in the evolution of the enormous number of fishes and an immense variety of reproductive strategies. Fish exhibit various types of sex determination, from genetic to environmental control, sex differentiation from hermaphroditism to gonochorism, age of puberty, from a few months to many years, fecundity, from a few to millions of eggs, internal or external fertilization, a wide range of egg sizes, some that float and others that sink or stick to substrates, uncared eggs scattered into the environment to parental care of eggs to live "birth" (ovoviviparity) [2-4]. The existence of these diverse reproductive strategies has important implications for finfish culture and broodstock management.

Finfish culture is the fastest growing food production industry in the world, and in 2005 a total of 28.3 million T of finfish were produced, which is around 20% of the world's fisheries production (aquaculture and capture fisheries) [5]. The number of species being cultured is also increasing rapidly, and 67 different cultured finfish species in 2005 had an annual production of over 10,000 T, which is twice the amount produced by the 28 species being cultured in 1980 [6]. One of the most important aspects at the basis of this continuing increase in the number of cultured species is our growing understanding of the complexities of the many different reproductive strategies of various fishes and how these behave in captivity. This is perhaps best demonstrated by the development of the culture of the catfishes (*Pangasius ssp*) in Vietnam. These catfishes do not mature in captivity, but during the 1990s hormone stimulation techniques were developed to induce ovulation/spermiation. This technology gave the control required to produce eggs, larvae and juveniles that formed the basis of an expanding aquaculture industry, producing 40,000 T in 1997 and 376,000 T in 2005 [6].

This chapter aims at giving a general vision of fish reproduction and the reproductive dysfunctions found in captive-reared fishes, and describe how this knowledge is used to develop treatments for the control of fish reproduction in aquaculture. This general view will be covered under four broad areas relevant to the

control of gonad maturation and spawning of fish in captivity. First, it will be described the normal gonadal development as can be expected under optimal (natural) conditions, particularly the morphology of gonad development and its endocrine and environmental regulatory mechanisms. Second, the reproductive dysfunctions that are observed in fish held in captivity; often the captive environment does not provide the environmental conditions that a species requires to complete maturation. Third, the available techniques for the stimulation of ovulation and spermiation, both environmental manipulations and hormonal treatments of reproductively dysfunctional fish held in captivity. Finally, the last sections will describe the application of adequate broodstock management protocols and hormonal therapies for the stimulation of fish reproduction in captivity for some of the main food fish species under production, both as a reference and indication of the possible strategies available to stimulate ovulation and spermiation in species considered candidates for new aquaculture developments.

2. THE REPRODUCTIVE CYCLE OF FISH

The reproductive cycle is an ensemble of successive processes from immature germ cells to the production of mature gametes, with the final purpose of obtaining a fertilized egg after the insemination with a spermatozoon. The process of gamete growth and differentiation is called gametogenesis, and leads to the formation of the female oocyte (oogenesis) or the male spermatozoon (spermatogenesis). Both the female and male gametes have a common origin in the population of embryonic primordial germ cells (PGC) that migrate during embryonic development to the place of gonad formation, the germinal epithelium. The PGC proliferate through mitotic divisions to form the gonia, which differentiate into oogonia or spermatogonia depending on the sex of the individual. With the last mitotic division, gonia enter meiosis and become oocytes or spermatocytes, thus initiating gametogenesis in adult animals [7-8].

In both males and females, the reproductive cycle involves two major phases, the phase of gonadal growth and development (gametogenesis) and the phase of maturation, which culminates in ovulation/spermiation and spawning. The release of mature gametes to the external environment (spawning) is a highly synchronized event, leading to fertilization of the egg and development of the embryo. The success of reproduction depends on the successful progression through every process of the reproductive cycle, which leads to the production of good quality gametes. This section describes some general features of reproductive physiology and gonadal development in fish.

2.1. Ovarian development in females: oogenesis, maturation and ovulation

The ovary of female fish is a bilateral elongated organ, localized in the abdominal cavity. The ovarian lobules are surrounded by the mesovarium and project posterior through a pair of oviducts that connect to the genital papilla, which opens to the external environment. The ovaries are compartmentalized by folds of the germinal epithelium that project transversally to the ovarian lumen, the ovigerous lamellae. In these lamellae, the oocytes undergo the various phases of gametogenesis, until mature ova (i.e., eggs) are released into the ovarian cavity or abdominal cavity (*e.g.*, salmonids) at ovulation and then to the external environment during spawning. Ovulated ova may remain in the ovarian/abdominal cavity for a period of time before spawning. There, they maintain their maturational competence (fertilizing capacity) for a certain period of

time, but if not spawned, the ova become “over-ripe” through a process of degeneration. This is an important consideration in cultured fish whose reproduction is based on manual egg stripping and artificial insemination, because stripping should be performed before over-ripping occurs. The lapse of time between ovulation and over-ripping varies greatly among fish, from minutes (*e.g.*, striped bass, *Morone saxatilis*) to days (*e.g.*, salmonids) and depends greatly on water temperature. In salmonids, which do not have a complete mesovarium and the oocytes are ovulated directly into the abdominal cavity, the ovulated ova can remain for several days with no evident over-ripping.

The germinal unit of the ovary consists of an oocyte surrounded by two layers of follicular cells. These follicular cells envelop the germ cell and offer structural and functional support to the developing oocyte, mediating the internalization of external molecules and synthesizing hormones and factors necessary for the differentiation, growth and survival of the oocyte. Each oocyte is surrounded by an inner mono-layer of granulosa cells and an outer mono-layer of theca cells [9]. Between the two follicular layers there is a thin basal membrane, which separates them. Also, a thick acellular envelop surrounds the oocyte (i.e., zona radiata), to which the granulosa cells are directly attached. The zona radiata develops progressively during gametogenesis, becoming increasingly thick and compact to constitute the egg chorion or egg shell.

The female reproductive cycle of fish is characterized by the specific process of vitellogenesis, the synthesis of vitellogenin (VTG), the precursor of the vitellin reserves of the egg [10-13]. The VTG is a lipophosphoglycoprotein synthesized in the liver under the stimulation of estradiol (E_2). It is released into the bloodstream and incorporated progressively into the growing oocytes, via receptor mediated endocytosis, were VTG is proteolytically cleaved into smaller components (phosvitin, lipovitellin and β-component), giving rise to the vitellin reserves of the egg, the yolk or vitellus [14]. The optimal accumulation and processing of VTG is of vital importance for the quality of the egg and further survival of the hatched larvae, as this constitute the sole nutritional reserves of the larva, until the initiation of external feeding, several days after fertilization. The VTG is a female specific protein and its circulating blood levels correlates well with the initiation and progression of the gametogenic period. These characteristics of the VTG molecule make the use of specific VTG immunoassays a very useful tool in aquaculture. Such VTG immunoassays are used for sexing broodstock in those species without external sexual dimorphism, because VTG detection in blood indicates clearly a female. The VTG immunoassays are also used to follow the stage of gonadal development, as VTG blood levels increase concomitantly with oogenesis [15-17].

Figure 1: The process of oocyte development and maturation in female fish. It initiates with the mitotic proliferation of the oogonia that undergo primary oocytes when entering to meiosis. The primary oocytes go through a primary growth phase (pre-vitellogenesis), which involves the appearance of pale material in the cytoplasm and formation of the two layers of surrounding granulosa and theca cells (i.e., follicular wall). The secondary growth phase (vitellogenesis) involves the synthesis and incorporation into the oocyte of vitellogenin (VTG), and is associated with a drastic increase in size. During vitellogenesis, new inclusions appear in the cytoplasm, such as the cortical alveoli (white circles), lipid globules (light grey circles) and yolk granules (dark grey circles) and the oocyte wall (i.e., zona radiata) and follicular wall become increasingly thick. At the end of vitellogenesis, the cytoplasm is filled completely with lipid globules and yolk granules at the onset of coalescence, the nucleus (germinal vesicle, GV) (black circle) is centrally located and a thick zona radiata surrounds the oocyte. At early maturation, lipid globules and yolk granules continue coalescence and the nucleus migrates to the animal pole (GV migration, GVM). As maturation advances, there is a massive coalescence of yolk granules and localization of the nucleus at a peripheral position. Final oocyte

maturation (FOM) is characterized by the dissolution of the GV membrane (GV break down, GVBD) and hydration of the oocyte. Oocytes are finally ovulated into the ovarian or abdominal cavity, and are released in the water during spawning. In this diagram, the cell sizes are relative.

The reproductive cycle of female fish can be divided into the period of oocyte growth (gametogenesis or vitellogenesis) and the period of oocyte maturation, along which the oocyte goes though different stages of development, before ovulation and spawning (Fig. 1) [10-13,18-19]. Oocyte development has been described in detail in a few species of fish [20-23]. Before initiation of the reproductive cycle, the immature ovary contains nests of oogonia along the ovigerous lamellae, which proliferate through mitotic divisions. At a certain moment, part of the oogonia population enters meiosis and become primary oocytes, which are arrested immediately at prophase I. This is the initiation of gametogenesis, and meiosis will not be resumed until final oocyte maturation (FOM). The primary oocytes go through a primary growth phase or previtellogenesis, which involves an increase in size, the appearance of pale material in the cytoplasm and the appearance of the granulosa and theca cellular layers (i.e., the follicle). This is a hormone-independent phase, before the period of E_2-induced VTG synthesis. The secondary growth phase, or vitellogenesis, is characterized by the synthesis and enormous accumulation of VTG and vitellin related proteins into the oocyte, resulting in a 10-fold increase in size. This period involves several transformation processes of the oocyte and associated follicular cells, which lead to the further classification of the vitellogenic period into different developmental stages. These transformations involve successive changes in both the oocyte cytoplasm and surrounding membrane and follicular cells. At early vitellogenesis, the oocyte is small (around 100 μm in diameter), with an opaque cytoplasm almost deprived of inclusions, except some oil droplets. With the progression of vitellogenesis, new inclusions appear in the cytoplasm, such as the cortical alveoli, lipid globules and yolk granules. The order of appearance of each type of inclusion is species-specific. These inclusions increase in size and number during vitellogenesis, promoting the increase of oocyte size. Also, the zona radiata, as well as the granulosa and theca layers become increasingly thick in order to support the rapid oocyte growth. At the end of the vitellogenic period (late vitellogenesis), the post-vitellogenic oocyte is characterized by a large transparent cytoplasm completely filled with yolk granules and lipid globules, a centrally located nucleus (or germinal vesicle, GV), and a thick, clearly striated zona radiata, enveloped by the granulosa and theca follicular layers.

After vitellogenesis, oocytes undergo FOM, with the resumption of meiosis and its advance to metaphase II, at which time the first polar body is released and the oocyte becomes a secondary oocyte [24]. At early maturation, lipid globules and yolk granules start coalescence and the germinal vesicle begins its migration to the animal pole (GV migration, GVM). As maturation advances, there is a massive coalescence of yolk inclusions and localization of the GV at a peripheral position. Final oocyte maturation is characterized by the dissolution of the nuclear membrane, a process called GV break down (GVBD). The transformation of the lipid and yolk inclusions modifies the ionic composition of the cytoplasm, causing a drastic incorporation of water inside the oocyte, through increases in osmotic pressure. This tremendous hydration is especially relevant in fishes producing pelagic eggs, and causes a rapid 2-3 fold increase in oocyte volume. After hydration, the follicular wall ruptures and the oocyte is ovulated into the ovarian/abdominal cavity and released to the water during spawning.

During the spawning season, post-ovulatory follicles (POF) can be found in the ovary. These are the empty follicular envelops that remain after the oocytes are released; under the microscope they have the aspect of folded structures, and disappear during ovarian reorganization a few days after ovulation. Atretic or apoptotic oocytes can also be found. These are oocytes that interrupted the process of vitellogenesis or FOM because of a failure in the hormonal regulation of the reproductive process. When the oocytes die, the vitelline envelop is fragmented and the hypertrophied follicular cells invade the ooplasm for phagocytosis. Follicular atresia appears under the microscope as a compact and well vascularized structure. The number of atretic oocytes increase along the pre-spawning and spawning season and it is a clear reflect of the success of reproduction of the female. It occurs in all species and relates to the appropriateness of the reproductive environment. Follicular atresia can occur at all stages of oogenesis and regulates the number of oocytes that advance through the reproductive process, affecting the fecundity of the species.

The determination of the stage of gonadal development in female breeders is an important tool in aquaculture. This can be determined by examination of biopsies of oocyte samples. The biopsy is performed in anesthetized females, by insertion of a cannula through the gonoduct and gentle aspiration of intra ovarian oocytes. The collected oocytes are observed under the binocular and classified according to their size, position of the GV (central, migrating or peripheral), degree of yolk granule coalescence, etc.; these classifications give a relative indication of the stage of gonad development of the females.

2.2. Testicular development in males: spermatogenesis, maturation and spermiation

The male gonad (testes) is also comprised of germinal and somatic tissue [25]. The germinal cells develop during spermatogenesis to give rise to the gametes, the spermatozoa. The somatic tissue of the testes forms the seminiferous tubules and supporting connective tissue, as well as specialized somatic cells, the Leydig and Sertoli cells [26]. These somatic cells offer structural and functional support to the germinal cells and play a crucial role in the production of hormones and other factors necessary for germ cell differentiation, development and survival. The Sertoli cells envelop the germ cells to form units called cysts or spermatocysts. The sum of all cysts constitutes the germinal epithelium of the testes. The Sertoli cells are attached to a basement membrane, which separates the germinal epithelium from the interstitial compartment. The interstitial compartment is formed by somatic tissue, in which the Leydig cells are located, between the seminiferous tubules. The Leydig cells, as the Sertoli cells, are also specialized endocrine cells, with an important role in the production of the necessary hormones for germ cell development.

Figure 2: The process of spermatozoa development and maturation in male fish. It initiates with the mitotic proliferation of the spermatogonia, first through slow division rates (spermatogonia A) and then through rapid division rates (spermatogonia B). Spermatogonia B emerge as primary spermatocytes, which enter to the first meiotic division; they become secondary spermatocytes that enter to the second meiotic division, leading to the formation of haploid spermatids. The spermatids differentiate into flagellated spermatozoa during spermiogenesis, which involves a drastic reduction in size (>80%) and formation of the flagellum. The flagellated spermatozoa are then released into the testicular lumen, were they undergo the process of maturation, by which they acquire the fertilizing capacity. The mature

spermatozoa are stored in the testes until they are released in the water during spermiation. In this diagram, the cell sizes are relative.

The process of spermatogenesis can be divided in three major phases, 1) the mitotic proliferation of the spermatogonia, 2) the meiotic division of the spermatocytes, and 3) the transformation of the haploid spermatids into flagellated spermatozoa (spermiogenesis). Through these, the germ cells go through different developmental stages, spermatogonia A and B, primary (2n) and secondary (1n) spermatocytes, spermatids and spermatozoa (Fig. 2). The presence and relative abundance of each of these classes in the testes is used as an indication of the degree of testicular development [27-28]. Before initiation of gonadal development, the immature testes contain spermatogonia (spermatogonial stem cells) that proliferate by mitotic divisions, through a self-renewal process. During this phase the population of stem cells in the testes increases in number. At a certain moment, some spermatogonial stem cells enter the process of spermatogenesis and are committed to produce spermatogonia. During the phase of mitotic proliferation of the spermatogonia (phase 1), each spermatogonium goes through several cycles of mitotic divisions, ranging from five to fifteen, depending on the species. During division, cytokinesis is incomplete and daughter cells maintain direct cytoplasmic bridges between them, remaining together in a cluster, called spermatocyst. This cluster of daughter cells is thus formed by a clone of spermatogonia, as they all come from a single original cell. Each cyst is enveloped by a wall of somatic Sertoli cells that maintain different clones separated from each other. During this phase of mitotic proliferation, the spermatogonia go first through a phase of slow division rate (spermatogonia A) and then through a phase of rapid division rate (spermatogonia B). The last mitotic division of spermatogonia B gives rise to primary spermatocytes, which will enter to the process of meiosis (phase 2). During phase two, the primary spermatocytes proceed to the first meiotic division, which involves DNA duplication and recombination of the genetic information, leading to the formation of secondary spermatocytes. They go rapidly to the second meiotic division, without DNA duplication, leading to the formation of haploid germ cells, the spermatids.

The spermatids enter the process of spermiogenesis (phase 3), in which the haploid spermatids differentiate into flagellated spermatozoa. This process does not involve cellular proliferation, but only cell transformation, which includes a drastic reduction in size (>80%) due to nucleus condensation and extrusion of the cytoplasmic content to the surrounding Sertoli cells. In addition, concentration of mitochondria, formation of a midpiece and formation of the flagellum take place at this time. In all but a few species of fish, there is no formation of the acrosome present in all other vertebrates, because fertilization is achieved through the micropyle of the fish egg. Once spermiogenesis is completed, the spermatocysts opens up by rupture of the Sertoli cell wall and the flagellated spermatozoa are released into the testicular lumen. Here, the spermatozoa will undergo the final process of maturation or capacitation, by which they acquire the fertilizing capacity (capacity of motility) [29]. Maturation occurs during sperm migration along the efferent duct and involves only physiological changes. Simultaneous to sperm maturation, the efferent duct produce a high amount of fluid (sperm hydration), leading to the formation of the milt, the fluid containing suspended spermatozoa. The released spermatozoa are stored before spawning and, depending on the species, the storage place is the tubular lumen, the efferent duct system or the germinal vesicles. Also, depending on the species, there are variations in the degree of maturation (fertilizing capacity) exhibited by the stored spermatozoa. For example, in salmonids, the intratesticular spermatozoa presents reduced fertilizing capacity as

compared to spermatozoa present in the efferent duct. This is important to consider when collecting sperm from a given species for fertilization experiments, as the fertilizing capacity of the collected sperm can vary depending on the location of sperm collection.

Structurally, the testes of teleost fish can be classified in two major types, tubular or lobular. The tubular testes is the most common among fishes. In this type, the spermatocysts are distributed throughout the testes, in a tubular structure and they do not move during the process of spermatogenesis. The lobular testes is found in some perciformes and atheriniformes and is characterized by the existence of lobules that blind-ended in the periphery of the testes; in these lobules the spermatogonia are restricted to the tips and the spermatocytes/spermatids move towards the efferent duct system during spermatogenesis.

2.3. Types of gonadal development

The high diversity of reproductive strategies of fish and that of inhabited environments is also reflected by the existence of a high variety in the types of gonadal development. This has important consequences to the fecundity and spawning characteristics of each species. The gonadal development in fish can be classified in three major types: synchronous, group-synchronous and asynchronous [11,19,30].

The synchronous type is exhibited by those species spawning only once in their life. This is the case of the lamprey (*Petromyzon spp*), the freshwater eel (*Anguilla spp*), some shad (*Alosa spp)* and Pacific salmons (*Oncorhynchus spp)*. In this type of ovary, all oocytes advance in synchrony through all the phases of gametogenesis, FOM and ovulation. Thus, only one type of developing oocyte class is present in the ovary. The group-synchronous type is exhibited by the seasonal spawners, those species that spawn one or more times during the annual reproductive season. In this type, a cluster of vitellogenic oocytes is recruited and advance synchronously through further stages of development, whereas the rest of the oocyte population remains arrested. The cluster of recruited oocytes will undergo maturation, ovulation and spawning. This type of ovarian development can be divided in two subgroups: single-batch and multiple-batch spawners. In the single-batch group synchronous species, only one batch of oocytes undergoes maturation every season and thus, they produce one single spawn per year (*e.g.*, rainbow trout, *Oncorhynchus mykiss*). The multiple-batch group synchronous species are able to repeat this process several times during the spawning season, with the recruitment of successive batches of oocytes and thus the production of several spawns per year. The number of spawning depends on the number of recruitments, *e.g.*, the European sea bass (*Dicentrarchus labrax*) producing 2-4 spawns per season. The asynchronous type of ovarian development is exhibited by those species that produce multiple spawns through an expanded period of time (several months), normally on a daily basis. This is typical of some tropical species, but also many Mediterranean fishes of the Sparidae family [31-33]. The oocyte population develops in an asynchronic manner and all classes of oocytes (from early vitellogenesis to late maturation) can be found in the ovary at any moment of the reproductive cycle. There are no batches of oocyte growth. This would represent the extreme of a multiple-batch group synchronous type of ovarian development, making the classification in one of those categories difficult for some species. In fact, the classification of the ovarian development just represents a continuum and all possible strategies between the two extremes are found in fish.

In respect to male fish, the development of the testes is somehow more homogenous and could be described as an asynchronous type of development for all species. Male fish use to be fluent on a daily bases through a long period of time, normally overlapping the spawning period of the females. At every moment, several classes of cell development, from immature spermatogonia to spermatozoa, can be found in the testes. At the full spermiation period, the testes are mostly occupied by mature spermatozoa, ready for spermiation, while early in the season, a high percentage of less mature spermatocytes is present.

3. ENVIRONMENTAL REGULATION OF FISH REPRODUCTION

The aim of reproduction is to have offspring that survive. It has been recognized for a long time that food availability and environmental conditions are "ultimate" factors that determine survival and hence how a species evolves through natural selection [34]. Food availability or flow of energy (energetics) has been acknowledged as the ultimate factors central to reproduction [35-36]. Food availability and the ability to store energy determine when a fish proceeds to the completion of maturation (Fig. 3). Experimentally it has been demonstrated that groups of fish fed low rations exhibited a reduction in the percentage of fish that complete maturation [37-39]. Theories suggest that fish have the ability, through a bio-chemical threshold, to ascertain what size and/or age conditions are optimal to complete maturation both during the first and subsequent maturation episodes [37,40]. Food availability for off-spring and hence off-spring survival determines the timing of reproduction. Food availability exhibits seasonal variation in the higher latitudes as well as lower latitudes in the tropics. Reproduction is timed to ensure that critical periods of feeding for the survival of the offspring, particularly larvae and juveniles, coincide with the seasonal periods of high food availability. For species in the high latitudes this is usually in the spring and for species in the lower latitudes this is often in relation to fluctuations in nutrient levels caused by changes in ocean currents, temperature cycles or weather cycles such as rainy seasons. Therefore, maturation is a complex process that must be perfectly timed to ensure that spawning or critical off-spring feeding periods coincide with seasonal highs in food availability that are months or even years after maturation was initiated.

Fish have evolved to entrain maturational development with the predictable but constantly changing environmental parameters (*e.g.*, photoperiod); these parameters cue the progress of maturational development and predict the approach of optimal conditions for offspring survival. It is perhaps not surprising that the predictable parameters are often the same environmental factors that drive the seasonal change, weather systems and changes in ocean currents which result in cycles of food availability. These predictable parameters are termed proximate factors and examples are photoperiod, temperature, food availability, lunar cycle, rainfall, currents and pressure (fig. 3). The degree to which these proximate factors have been studied is variable and therefore our true understanding of the importance of the factor is also variable ranging from a complete understanding of the factors role to circumstantial evidence that an aspect of maturation was repeatedly observed to coincide with a change in the proximate factor.

Figure 3: Diagram of environmental factors affecting a fish.

Perhaps the most important proximate factor is photoperiod. The role of photoperiod as a proximate factor has been comprehensively described for the

salmonids and in particular the rainbow trout, through studies that examined the effect of natural and altered photoperiods on reproduction when other proximate factors such as temperature were maintained constant. From this type of studies it was demonstrated that photoperiod entrained an endogenous rhythm that controlled all aspects of maturational development, i.e. the entire brain-pituitary-gonad axis [41-42]. Therefore, in the rainbow trout the increasing spring photoperiod was shown to entrain the decision to proceed to the completion of maturation and in turn the start of vitellogenesis/spermatogenesis, the passage of photoperiod from spring to summer to autumn entrained the progress of vitellogenesis/spermatogenesis and the decreasing autumn photoperiod entrained final maturation, ovulation and spermiation. Photoperiod probably plays an important role in the timing of reproduction of most temperate fish species and has been shown to influence the timing of maturation in many species from a wide range of families that inhabit both temperate latitudes, such as the Atlantic salmon (*Salmo salar*, family: Salmonidae), European seabass (*Dicentrarchus labrax*, Percichthyidae) [43], gilthead bream, (*Sparus aurata*, Sparidae) [31], red drum (*Sciaenops ocellatus*, Sciaenidae) [44], Atlantic cod (*Gadus morhua*, Gadidae) [45], Atlantic halibut, (*Hippoglossus hippoglossus*, Pleuronectidae) [46], sole (*Solea solea*, Soleidae), turbot (*Psetta maxima*, Scophthalmidae) [47] and tropical latitudes, such as the Nile tilapia (*Oreochromus niloticus*) [48], grey mullet (*Mugil cephalus*, Mugilidae) [49], catfish (*Heteropneustes fossilis*, Heteropneustidae) [50] and common carp (*Cyprinus carpio*, Cyprinidae) [51-53]. However, the aspects of maturational development entrained by the different phases of the photoperiod cycle and the interaction with other proximate factors will depend on the reproductive strategy of each species.

Temperature also plays an important role as a proximate factor and many species, particularly tropical and sub-tropical species appear to time spawning in relation to changes in temperature. However, the role of temperature is not clear and can be argued to be act as a controlling or entraining factor such as photoperiod or a permissive factor that has a direct effect on the biological processes, but which is not actually used as a cue with which an organism times maturational development [42]. This unclear situation is partly caused by the lack of studies that have examined in isolation the interaction of temperature and maturation, with other proximate factors maintained constant, as studies have with photoperiod. However, despite this poor understanding, the importance of temperature in the maturational process can not be disputed. Most species examined have an optimum temperature profile for the various stages of maturational development and generally temperatures below this optimal range will delay maturation and temperatures above accelerate maturation [54]. Temperatures that are extremely different from the optimal temperature range stop gametogenesis and induce atresia. In particular these temperature effects have been observed in relation to spawning. For example, the dab (*Limanda limanda*) in the North Sea has been observed to mature in relation to seasonally changing temperatures, gametogenesis from October to January and spawning as the temperature begins to rise from February to April [55]. Spawning time of different stocks of dab was progressively later the further north the stock was found and spawning time was correlated with progressively later latitude dependant rises in temperature [54]. These observations have given rise to findings that spawning can be predicted from the temperature profile. For example, Baynes et al. [56] demonstrated that for sole (*Solea solea*) there existed a positive correlation (r=0.9) between winter temperature and the time that spawning started in the spring and Rothbard and Yaron [57] described how in Israel degree days

after the temperature rises above 15ºC are used to predict when carp are in spawning condition.

Other environmental parameters which have been observed to coincide with aspects of maturational development include food availability, lunar or tidal cycles, rainfall, currents and pressure. The knowledge of these sorts of parameters is based almost entirely on observations. Such observations, offer little explanation as to the parameters role in the timing of maturation and the usefulness of such parameters in aquaculture is questionable, when the difficulty or impossibility of manipulating or reproducing these parameters is considered. One explanation could be that many of these parameters are final cues and fish mature to late stages of vitellogenesis/spermatogenesis in relation to photoperiod and/or temperature and await a direct effect to cue final maturation and spawning. However, these parameters do highlight the diversity and complexity of reproductive strategies that have evolved. Some interesting examples include, timing of spawning of pelagic fish to coincide with plankton blooms [54], the spawning of Indian carps in relation to heavy monsoon rain and floods [58], timing of spawning with changes in currents on the Californian coast [59] and the association of captive seabream spawning with the lunar cycle [60].

4. HORMONAL REGULATION OF FISH REPRODUCTION

The reproductive cycle is regulated by a cascade of hormones along the brain-pituitary-gonad (BPG) axis, the so-called reproductive axis (fig. 4). In this axis, the pituitary gonadotropins (GTHs), Follicle-Stimulating Hormone (FSH) and Luteinizing-Hormone (LH) are the key players in the endocrine control of reproduction. The secretion of the two GTHs is controlled by the brain via the stimulatory action of the Gonadotropin-Releasing Hormone (GnRH). This neuropeptide is the primary system regulating reproduction, acting as an integrator of external information (*e.g.*, environment) and sending neuroendocrine inputs for the regulation of the reproductive axis. The GnRH acts directly at the pituitary gland to stimulate FSH and LH secretion that are released into the bloodstream to act on the gonad, where they stimulate the synthesis of gonad steroid hormones, which are the ultimate effectors of gonadal development.

Figure 4: The brain-pituitary-gonad (BPG) axis, showing the critical hormones involved in the regulation of fish reproduction. The reproductive process initiates in the brain, which integrates external information (e.g., environment) and respond with the activation of the Gonadotropin-Releasing Hormone (GnRH) system. The GnRH stimulate the synthesis and release of the pituitary gonadotropins (GTHs), Follicle-Stimulating Hormone (FSH) and Luteinizing-Hormone (LH), which act on the gonad (ovary or testes) to stimulate the synthesis of sex steroids, the ultimate effectors of gonadal development. The sex steroids play additional roles over non gonadal tissues, mainly feedback actions on the brain/pituitary and in females, the stimulation of vitellogenin (VTG) synthesis in the liver.

At initial stages, GTH stimulation (mainly FSH) induces the secretion of androgens (e.g., testosterone (T) and 11-ketotestosterone (11KT)) in males and estrogens in females, which act concomitantly with FSH in the control of gametogenesis. The estrogen estradiol-17β (E_2) plays an additional important role in female gametogenesis, with the stimulation of VTG synthesis from the liver. Thus, the period of gametogenesis is characterized by high blood levels of FSH and increasing levels of androgens in males, and increasing E_2 and VTG in the females. At the end of

gametogenesis, secretion of LH from the pituitary induces a shift in the steroidogenic pathway of the gonad, stimulating the synthesis and secretion of progestin-like steroids, the maturation-inducing steroids (MIS). The concomitant action of LH with the MIS stimulates the process of gonadal maturation. This period is characterized by decreasing blood levels of FSH and androgens/estrogens and increasing blood levels of LH and MIS. Once gonadal maturation is completed, the brain GnRH system stimulates a high surge of LH secretion from the pituitary, which induces ovulation in the females, whereas in the males, relatively stable but elevated levels of LH induce spermiation (fig. 5) [61-63]. The GnRH-induced pre-ovulatory LH surge in the plasma is essential for successful ovulation. In fact, the demonstration that this characteristic LH surge was absent in captive fish that failed to ovulate, but not in wild fish ovulating spontaneously, set up the bases for the development of hormone-based spawning induction therapies in aquaculture [64-65].

Figure 5: Evolution of the endocrine and gonadal changes associated to the reproductive cycle of female and male fish. The top half of the diagram shows the pituitary hormone secretions and plasma hormone concentrations; the bottom half shows the correlated stages of oocyte and sperm development. At initial stages, pituitary FSH stimulation induces gonadal secretion of estrogens in females (estradiol (E_2)) and androgens in males (11-ketotestosterone (11KT)) that regulate gonad development. In females, E_2 plays an additional role on the liver, stimulating VTG synthesis (vitellogenesis). The period of gametogenesis is characterized by high blood levels of FSH and increasing levels of androgens in males, and E_2 and VTG in females. At the end of gametogenesis, pituitary LH secretion induces the synthesis of maturation-inducing steroids (MIS), which regulate the process of gonadal maturation; this is characterized by decreasing blood levels of FSH and androgens/estrogens and increasing blood levels of LH and MIS. At completion of maturation, a GnRH induced LH surge stimulates ovulation, spermiation and spawning.

The success of reproductive maturation and viable gamete release depends on the correct functioning of all components of the reproductive axis throughout the entire reproductive cycle, from gametogenesis to spawning. The synchronized secretion of GnRH, GTHs and steroids through the reproductive cycle and their coordinated action is essential for successful spawning. The stress associated with captivity or the absence of appropriate environmental conditions in culture facilities may act on the brain inhibiting neuroendocrine secretions, and thus blocking the reproductive axis, and inhibiting reproductive success.

4.1. Brain Gonadotropin-Releasing Hormone (GnRH)

The brain is the highest level of the reproductive axis and acts as the director of reproduction, integrating external and internal information and responding with neuroendocrine signals. The primary neuronal system in the regulation of reproduction in all vertebrates is the GnRH. This is a neurohormone synthesized in specific areas of the brain, from where the GnRH cells project neuronal fibers directly into the pituitary. This system is unique in fish, since in higher vertebrates GnRH neurons do not project directly to the pituitary, but instead end into the median eminence and GnRH is released into a portal system, from where it reaches the pituitary gland. The GnRH is released in close vicinity to the gonadotropic cells, binds to specific membrane receptors and stimulates the synthesis and release of both FSH and LH. Due to its crucial role in the integration and regulation of the neuroendocrine signaling governing reproduction, the

GnRH system has been the focus of intensive research in reproductive biomedicine, for both basic research and applied uses of GnRH derived drugs for treatment of reproductive disorders [66-77].

The GnRH is a decapeptide that was first discovered in the brain of a mammalian species, and originally named Luteinizing-Hormone Releasing Hormone (LHRH), because of its LH-releasing activity [78-79]. It was also later named mammalian GnRH (mGnRH), a more appropriate nomenclature after the demonstration of its stimulatory action on both FSH and LH secretion. Since the characterization of the first GnRH, other GnRH forms have been isolated and characterized from the brain of other species, and up to date 24 different GnRH forms are known [77]. From them, 14 variants have been found in vertebrates, 9 in tunicates [80] and 1 in a cephalopod mollusk [81]. All GnRHs are decapeptides (except the octopus GnRH which is a dodecapeptide), with slight variations in their amino acid sequence. Each newly identified GnRH has been named after the species in which it was discovered first (table 1).

Table 1: Primary structure of the 24 known native GnRH forms. The first discovered (mGnRH) is taken as the reference. Octopus GnRH is the only variant with 12 amino acids, presenting an Asn-Tyr insertion at the N-terminus. Medaka GnRH (mdGnRH [90]) is also known as pejerrey GnRH (pjGnRH [96]).

In addition to the multiplicity of GnRH variants, an important finding was the demonstration that most vertebrate species express more than one GnRH form in the brain. As a general rule, two GnRHs are expressed simultaneously in the brain of a given species, localized in different regions of the brain and apparently exerting distinct biological functions. One GnRH system is directly involved in the regulation of pituitary secretion (i.e., the hypophysiotrohic system), whereas the other GnRH system does not. Fish are unique among vertebrates, because some teleost species have been found to express three GnRH forms in the brain [77]. In these species, the third GnRH system is related to the hypophysiotrophic GnRH system and probably both work coordinately in the regulation of pituitary secretion.

Due to the increasing number of GnRH forms and proposed names, the GnRH nomenclature has become somehow confusing. Recently, a new and more appropriate nomenclature for the GnRH family has been proposed, based on phylogenetic and neuroanatomical data. This nomenclature grouped all GnRHs in three main types, called GnRH-1, GnRH-2 and GnRH-3 [97-98]. The GnRH-1 would refer to the GnRH form directly involved in the regulation of pituitary GTH secretion, the classical hypophysiotrophic system. In the old nomenclature this GnRH-1 would correspond to one of eleven GnRH variants, depending on the species: mGnRH or gpGnRH in mammals and primitive fish (e.g., *Anguilla spp*), cGnRH-I in birds and reptiles, frGnRH in amphibians and sGnRH, ctGnRH, sbGnRH, hgGnRH, mdGnRH, whGnRH or dfGnRH in fish. These GnRHs have in common their biological function and brain distribution. They are synthesized by neurons located in the hypothalamus, from where they send numerous projections to the pituitary, where GnRH-1 is released to stimulate GTH secretion.

The GnRH-2 refers to the form synthesized by neurons located in the midbrain. These neurons do not send projections to the pituitary, where this form is absent, and therefore, it is believed that GnRH-2 does not have a direct role in the control of pituitary GTH secretion. In contrast to the previous system, the GnRH-2 corresponds

always to the cGnRH-II variant of the old nomenclature. All the species studied, from fish to mammals, express cGnRH-II in this area of the brain. This ubiquitous system, highly conserved in the evolution, should have important functions, but to date there are no clear evidences of the specific biological functions of GnRH-2, although a potential role in the regulation of reproductive behavior has been claimed [99-100]. The GnRH-3 system is unique in teleost fishes. It is clearly related to the GnRH-1, considering phylogenetic and morphological data. The localization of GnRH-3 neurons varies slightly between species, but normally overlaps with that of the GnRH-1 neurons. Nevertheless, GnRH-3 is always predominant in anterior regions (olfactory bulbs), whereas GnRH-1 predominates in the preoptic area (hypothalamus). The GnRH-3 neurons send a few projections to the pituitary, suggesting a potential involvement in the co-regulation of pituitary secretion [77,101-102].

The multiplicity of GnRH variants and simultaneous presence of several GnRHs in the brain has raised important questions on their specific biological functions but also on the development of specific GnRH-derived analogues for therapeutic applications. It has been demonstrated that all GnRH forms stimulate LH secretion. Research on GnRH structure-activity has been directed towards the development of GnRH agonists (GnRHa), in which modifications of the GnRH structure could lead to increased bioactivity with respect to the native form. The highly conserved regions of the decapeptide structure, the NH_2-terminus (pGlu-His-Trp-Ser), the COOH-terminus (Pro-GlyNH_2) and the amino acid at position six, are indicative of the importance of these sequences in the bioactivity of the molecule, in regard to enzymatic resistance, receptor binding and activation. Based on these studies, thousands of GnRHa´s and antagonists have been developed for therapeutic applications in the control of reproductive disorders.

The stimulatory action of GnRH on GTH secretion is dependent on the presence of GnRH receptors (GnRH-R) in the membrane of the pituitary gonadotrops. Similarly to the situation for the GnRH ligands, multiple GnRH-R´s are expressed in a single species. In mammals, two types of GnRH-R, named type I and type II, have been identified, which display ligand specificity for each GnRH variant [71]. In fish, multiple GnRH-R´s have been identified and, in contrast to mammals, they do not show ligand specificity. All fish GnRH-R´s display higher affinity for cGmRH-II than for sGnRH or the hypophysiotrophic form [70,103]. Expression levels of the GnRH-R genes in the pituitary show a seasonal pattern, which is an important factor influencing the seasonal responsiveness of the pituitary to GnRH stimulation. Highest levels of GnRH-R and thus highest responsiveness of the pituitary occur at the pre-spawning period, whereas lowest GnRH-R levels are found during the resting period and early stages of gonadal development. This is critical not only for the natural development of the reproductive cycle, but also when applying hormonal therapies, as this affects greatly the efficiency of GnRHa-based hormonal treatments, depending on the moment of the cycle when the treatments are applied.

In addition to the primary GnRH stimulatory system, GTH secretion is under the influence of a brain inhibitory tone, the dopaminergic system [104]. Neurons secreting dopamine (DA) exert an inhibitory action on both the brain and pituitary. Over the GnRH system, DA inhibits GnRH synthesis and GnRH release. In the pituitary, DA down-regulates GnRH-R and interferes with the GnRH signal-transduction pathways, inhibiting GnRH-stimulated LH secretion from the pituitary [105]. A dopaminergic inhibition on LH release has been demonstrated in all vertebrates, including amphibians, birds, mammals and humans [106-108]. Its intensity and moment of action may differ greatly between species, depending mostly on different reproductive strategies. In fish,

a dopaminergic inhibition of reproduction has been demonstrated in cyprinids, silurids, salmonids, tilapia (*Oreochromis spp.*), European eel (*Anguilla anguilla*) and grey mullet (*Mugil cephalus*) [109-114]. In these species, DA inhibits strongly the pre-ovulatory GnRH-stimulated LH surge and thus, ovulation and spawning; it also seems to be involved in the inhibition of puberty. In contrast, an active DA inhibitory system seems to be very weak or absent in most marine fishes.

Although GnRH is the primary regulator of reproduction, the brain synthesizes other neurohormones and neurotransmitters that have been shown to stimulate LH secretion and participate in the regulation of fish reproduction, being the most relevant the neuropeptide Y (NPY) and the neurotransmitter γ-amino-butiric acid (GABA) [61,115-118]. The NPY is involved in the regulation of the nutritional status of the fish; NPY neurons exert stimulatory actions on both GnRH and GTH and seem to play and important role in mediating interrelationships between nutrition and reproduction. The GABA is the most relevant neurotransmitter of the brain, also in mammals, and exerts a stimulatory action over LH secretion. It seems that the profusion of GABA neurons in the brain plays an important role interconnecting different neuronal systems, synchronizing and fine tuning neuronal secretions from different systems. Other neuronal systems have also shown to exert some LH stimulatory action, but they are of minor relevance. In general, all these neuronal systems act over both the GnRH neurons and/or the gonadotrops stimulating GnRH secretion, GnRH-R levels and FSH/LH synthesis and release. They can also act over the dopaminergic neurons, inhibiting DA secretion and thus exerting a stimulatory action on LH release. This neuronal network seems to fine tune the correct functioning of the primary GnRH-GTH endocrine system.

4.2. Pituitary Gonadotropins (GTH)

The pituitary or hypophysis is a major endocrine gland localized in the ventral part of the brain and is responsible for the release of the GTHs, in addition to several other hormones involved in growth, metabolism and stress adaptation. The two pituitary GTHs, FSH and LH, together with the Thyroid-Stimulating Hormone (TSH) and the placental chorionic gonadotropin (CG), constitute a family of structurally related molecules, the glycoprotein hormones [119]. They are heterodimeric proteins, constituted by a common α subunit, non-covalently linked to a hormone-specific β subunit, which confers the biological specificity to the hormone. Each glycoprotein subunit is encoded by a different gene. Early after synthesis, the peptide chain is folded, glycosilated and assembled to form the dimeric conformation, required for the biological activity of the hormone. The bioactivity of the GTHs depends on the duration of time that the hormone is present in the circulation (half-life), the binding to specific receptors and the activation of intra-cellular signal transduction mechanisms that leads to the biological response [120-121]. The half-life of the GTHs in the bloodstream is determined mainly by its degree of glycosilation. This is one of the main reasons for the use of human CG (hCG) in the hormonal treatment of several reproductive disorders, including spawning induction protocols in fish. The hCG is the highest glycosilated GTH and thus, it presents higher resistance to degradation than any other glycoprotein, thus having long acting effects. The stimulation of the target cells also depends on GTH binding and activation of specific membrane receptors. There are two types of GTH receptors, exhibiting ligand specificity for each gonadotropin. The hormone specific β subunit determines the specificity of the binding (FSH for the FSH-R, LH for the LH/CG-R), preventing interaction of a given GTH with the receptors of other glycoproteins. The human CG (hCG) binds to the same receptors as LH. This is

an important reason that justify the use of hCG as an hormonal treatment for spawning induction in fish, as hCG can display LH related functions and thus induce ovulation and spermiation in captive fish (see following sections).

Research in the field of fish GTHs has followed that in mammals. For many years, it was believed that the fish pituitary produced a single GTH responsible for the control of all aspects of reproduction, in contrast with higher vertebrates. This single GTH in fish had characteristics similar to the LH of higher vertebrates. In 1988, two distinct GTHs were purified and identified for the first time from the pituitary of a fish species, and named GTH-I and GTH-II [122]. The similarity of these fish GTHs with tetrapod FSH and LH was further established through molecular, biochemical and immunological techniques [123]. Thus, when reviewing the bibliography referred to fish reproduction, one has to be aware of the nomenclature used for fish GTHs. The bibliography before 1988 cite only the name "GTH", which refer to a hormone that was later confirmed to correspond to LH. During the next decade, information regarding the fish GTHs, refer to the names "GTH-I and GTH-II" (or "GTH-1 and GTH-2"), GTH-II being the previously known GTH (LH-like hormone) and GTH-I the newly discovered hormone. This nomenclature has now been abandoned and recent fish bibliography refers to the names "FSH and LH", standardizing the nomenclature with that of all vertebrates, FSH being homologous to the previous GTH-I and LH homologous to the previous GTH-II.

Information on the structure-activity and biological functions of LH in fish reproduction is much more extensive than that of FSH [105,124]. This is because immunoassay methods for analyzing LH secretion in fish have been available for many decades, while FSH immunoassays were only available since 1988, and almost exclusively limited to salmonid species [124]. The initiation of the reproductive cycle is characterized by increased FSH levels, which are maintained high during gametogenesis, whereas LH levels remain undetectable. During gonadal maturation, FSH levels decline and LH increase, showing a sharp LH peak prior to ovulation. The recent development of molecular tools has allowed the analysis of FSH and LH gene expression levels in several fish species, obtaining information on the biological functions of both hormones in a broader range of fishes. In salmonid species, showing single-batch group synchronous ovarian development, mRNA levels of βFSH increase during early gametogenesis while βLH predominates during FOM [125]. Information in non-salmonid species shows a slightly different picture. In the gilthead seabream (*Sparus aurata*), with asynchronous ovarian development, both βFSH and βLH are expressed throughout the year, increasing both during the reproductive season [126]. In other non-salmonid species, exhibiting multiple-batch group synchronous or asynchronous ovarian development, such as the blue gourami (*Trichogaster trichopterus*), red seabream (*Pagrus major*), European seabass (*Dicentrarchus labrax*) and stickleback (*Gasterosteus aculeatus*), FSH and LH gene expression levels are found throughout the reproductive cycle, although in most cases FSH synthesis is advanced with respect to that of LH [127-130]. The general view is that FSH controls mainly early stages of gametogenesis, while LH regulates FOM, ovulation and spermiation. Nevertheless, it becomes clear that there are important differences between fish species, most probably related to different patterns of gonadal development and different reproductive strategies.

4.3. Gonad steroids

The gonad is the tissue for generation of gametes but also a major endocrine organ, specialized in the synthesis of sex steroid hormones. These steroid hormones are the final endocrine effectors of gonadal development, in coordination with the pituitary GTHs [2,9,131-132]. Steroidogenesis takes place in the somatic cells of the gonad, the granulosa and theca cells in the ovary and the interstitial Leydig cells and Sertoli cells in the testes. The major steroid hormones in the regulation of fish gametogenesis are the estrogen E_2 in females and the androgen 11KT in males. In mammals, the main estrogen in females is also E_2, but the main androgen in males is T, instead of 11KT, and to a lower extent dihydrotestosterone (DHT). The fish ovary also synthesizes T, which plays other reproductive related functions. Similarly, males also synthesize the E_2, but this is found in much lowers levels than in females. The testes of male fish produce other androgens than 11KT (*e.g.*, T), which exert complementary functions during testicular development [26].

In addition to their role in regulating gonadal development, sex steroid hormones also exert both positive and negative feedback on the brain-pituitary axis and thus, regulating GTH release. A major positive action of the steroids is to enhance pituitary responsiveness to GnRH, probably by stimulating GnRH-R. A major negative action of these steroid hormones is exerted through the dopaminergic system, increasing DA turnover and thus enhancing the DA inhibitory tone over GTH secretion. In this way, the brain is constantly informed about the evolution of gonad development, through the action of the fluctuating circulating levels of steroids during the reproductive cycle [133].

4.3.1. Steroids regulating female oogenesis and maturation

In females, E_2 acts in coordination with the pituitary GTHs in the regulation of oocyte development. In the ovary, steroidogenesis is a two-cell biosynthetic process, in which the outer theca layer synthesizes steroid precursors that are transported into the granulosa cells, where they are transformed into derivates. During vitellogenesis, the theca cells synthesize T that is converted into E_2 in the granulosa cells, by the action of the enzyme aromatase. During vitellogenesis, E_2 exerts two main functions, one in the gonad regulating oocyte development and one in the liver stimulating the synthesis of VTG and other yolk related proteins.

Once vitellogenesis is completed, pituitary LH secretion induces a shift in the steroid biosynthetic activity of the ovary with a reduction in T and E_2 production and enhancement of the synthesis of MIS. This is caused by reduction of aromatase activity and increased activity of enzymes of the MIS pathway. There are two major MIS identified in fish, 17α,20β,dihydroxy-4-pregnen-3-one (17,20β-P or DHP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S). They both probably act as MIS is most fishes, but normally one of them is the predominant MIS for a given species. The 17,20β-P is the major MIS in several salmonid and non-salmonid species, while 20β-S is the major MIS in Atlantic croaker, spotted sea trout, striped bass and black porgy [134]. The synthesis of MIS is also a two-cell process, by which the precursor 17α-hydroxyprogesterone is synthesized in theca cells and converted into 17,20β-P in the granulosa cells, by the enzyme 20β-hydosysteroid dehydrogenase. The MIS together with pituitary LH secretion regulate gonadal maturation. The action of MIS on FOM is not direct, but mediated by the complex interaction of different factors, including prostaglandins (PGE1, PGE2, PGF1α, PGF2α), insulin-like growth factors (IGF-I and IGF-II), activin B and other signal transduction pathways [135]. It is the rise of MIS rather than the reduction of other steroids which is responsible of inducing FOM. That

is why in many fish species blood levels of estrogens remain high during gonadal maturation. In multiple spawners and some single spawners, plasma levels of MIS correlates well with the maturation cycle and E_2 levels are maintained high through the entire period of maturation.

During maturation, the oocyte goes first through a phase of oocyte maturational competence (OMC) before FOM can be achieved. During this phase, the oocyte acquires the competence to mature, including LH stimulation of follicle cells to produce necessary factors for MIS biosynthesis (enzymes, etc.) and the stimulation of germ cell capacity to respond to MIS (MIS receptors, etc.). During OMC, which is developed without MIS secretion, the first signs of FOM are evident (lipid globule coalescence and GVM). During FOM, LH dependent MIS secretion from follicular cells acts over membrane receptors in the oocyte to undergo final coalescence of yolk granules, GVBD and the resumption of meiosis.

4.3.2. Steroids regulating male spermatogenesis and maturation

Testicular spermatogenesis and maturation is also regulated by pituitary GTH secretion, but the action of the steroids secreted by the testes has a stronger influence. The androgen 11KT is the major regulator of spermatogenesis, while MIS regulates sperm maturation. Both steroids are synthesized by the somatic cells of the testes under GTH stimulation. The LH is mainly involved in the stimulation of androgen production in Leydig cells, whereas FSH seems to exert more complex functions in the male testes, stimulating androgen production in Leydig cells and regulating Sertoli cell activity during spermatogenesis. Although the regulatory mechanisms of FSH are mostly unknown, possible functions of FSH in the testes are, the stimulation of Sertoli cell proliferation and differentiation and synthesis of growth factors, which act as autocrine and paracrine factors involved in Sertoli cell proliferation and differentiation and germ cell development.

Before initiation of spermatogenesis, spermatogonial stem cell renewal seems to be regulated by E_2 acting on Sertoli cells. At a certain moment, secretion of pituitary GTHs (mainly FSH) induces a switch from spermatogonial self-renewal to spermatogonial proliferation, which represents the initiation of spermatogenesis. The FSH acts on Sertoli cells and stimulate 11KT biosynthesis through activation of specific enzymes (11β-hydroxylase and 11β-hydroxysteroid dehydrogenase). From then on, 11KT regulates the full process of spermatogenesis, an action that is mediated by growth factors secreted by the Sertoli cells. In males, FSH levels are high at early spermatogenesis, increase to maximum levels during the rapid testicular growth phase and then decline after spawning. On the other hand, LH is very low during early spermatogenesis, start increasing during the rapid testicular growth phase and peaks during spawning. As spermatogenesis advances, LH becomes important in supporting 11KT production. After completion of spermatogenesis, secretion of LH from the pituitary induces a shift in the steroiodogenic pathway of the testes leading to the production of MIS, which in turn regulate sperm maturation. During maturation, 17α-hydroxyprogesterone synthesized in Leydig cells is converted to MIS in the spermatozoa due to the activity of 20β-hydosysteroid dehydrogenase. The action of MIS on sperm maturation is not direct on the sperm, but via activation of specific enzymes that increase seminal plasma pH, which in turn induces the spermatozoa capacitation. In males, androgen production remains high through the entire period of sexual maturation, even while MIS levels are high.

5. REPRODUCTIVE DYSFUNCTIONS IN CAPTIVE FISH

As already mentioned in previous sections, there is a significant variation in fish reproductive strategies and types of gonad development. During their reproductive cycle, which can last for days, months or years depending on the species, fish experience a variety of external influences. In their natural habitats, the endocrine reproductive axis of the fish functions correctly and reproduction develops successfully, with spawning taking place at the moment when the fish detect that the external conditions are the most appropriate for the survival of the offspring and of course, its own survival. Unfortunately, the situation may change drastically when fish are reared in captivity and reproduction is somehow affected by the captive conditions. In fact, all fish species held in captivity exhibit some degree of reproductive dysfunction. And, it is normally the females who exhibit more serious reproductive problems. These dysfunctions depend on the species and can vary from a total absence of spawning to significant reductions in the quantity and quality of the eggs and sperm produced.

The reproductive problems detected in captive fish are derived from two causes, the stress associated with captivity and the absence of appropriate environmental signals permissive for reproduction [136]. The action of one of these factors or the combination of both underlies the total or partial inhibition of reproduction in captivity. Thus, the primary task of a broodstock manager will be to minimize the negative effects of these two parameters, in order to obtain the best reproductive performance of the cultured breeders. The negative influence of stress should be minimized by appropriate broodstock management (fish manipulation, fish care, prophylaxis, etc) and adequate culture conditions (tank design, water supply, light intensity, etc). This should be adapted to each species, considering that resistance and adaptiveness to stress varies greatly among species. The second parameter, the absence of appropriate environmental signals, is somehow much more difficult to solve. The broodstock manager should learn as much as possible about the reproductive biology of the species in its natural habitat and try to adapt the culture conditions to the natural situation. For many species, it is almost impossible to mimic the environmental conditions that the fish experience during their reproductive season (*e.g.*, migration to the spawning grounds). The complexity of environmental factors to which the fish is exposed during the whole reproductive period is basically unknown for most species and would, anyway, most probably be difficult to reproduce in culture conditions. This is normally more feasible for non-migratory species inhabiting stable habitats, but becomes more complex or unfeasible for long-distance migratory species (e.g., *Anguilla spp*, *Seriola spp*, and *Thunnus spp*). In any case, the better we provide the required environmental signals, the less reproductive problems will be exhibited by the breeders. If reproductive disorders persist even after taking maximum care to reduce the negative effects of these parameters, then the use of hormonal treatments can overcome reproductive problems, as demonstrated for many cultured fishes [62,64-65,137-139].

The sensory and endocrine system of fish has evolved to recognize when external and internal conditions are optimal for reproductive development and has the capacity to, 1) under optimal conditions maintain reproductive development to completion and spawning, 2) under sub-optimal conditions arrest development at a particular stage and postpone gamete production for improved conditions, or 3) under non-optimal conditions abort reproductive development, reabsorb nutrients invested in the gonad and return the gonad to a resting stage. These pathways have developed to ensure survival of both offspring and the parents. Under optimal conditions the sensory and endocrine system of the parents has the ability to recognize that the possibility of

offspring survival is high and therefore, the parents risk personal survival to invest energies in reproduction and spawning. However, when conditions are not optimal for spawning the endocrine system has the ability to recognize that the risk to parental survival of investing energy in maturation may not be rewarded with survival of the offspring and maturation is arrested or in extreme conditions aborted.

Figure 6: Major reproductive dysfunctions observed in captive female fish. They are classified in three main types (indicated by X), 1) the inhibition of vitellogenesis, 2) the inhibition of oocyte maturation, which cause atresia of post-vitellogenic oocytes and, 3) the inhibition of spawning only, with ovulated oocytes retained in the ovarian or abdominal cavity. Each type is physiologically different, but the end consequence is similar for the aquaculturist, the absence of spontaneous spawning in the tank. The application of hormonal treatments has effectively resolved reproduction in many species exhibiting dysfunction type two. Solution of dysfunction type one is under investigation, whereas reproduction of species with dysfunction type three can be achieved through artificial fertilization, after manual stripping of eggs and sperm.

In captive broodstock, the females normally exhibit more serious reproductive problems than the males; female dysfunctions can be classified in three main types (fig. 6). The first type is the inhibition of vitellogenesis. In this species, reproduction is blocked at very early stages of development, e.g., *Anguilla spp* and sometime *Seriola spp* [140] (Mylonas et al., 2004b). Physiologically, this is the most serious reproductive disorder, as the endocrine reproductive system of the fish has not functioned at any moment of the reproductive process. The second type of reproductive dysfunction is the inhibition of the process of FOM. In species exhibiting this problem, vitellogenesis is completed correctly, but post-vitellogenic oocytes are unable to undergo FOM and become atretic. The degree of inhibition varies depending on the species and even on the environmental conditions of each specific reproductive season. Atresia can affect the whole population of post-vitellogenic oocytes of the gonad, causing the total absence of spawning, or may affect only part of the post-vitellogenic oocytes, which finally causes a reduction in the number of eggs released. The diminished egg production can be slight, or can be dramatic, causing only sporadic spawning of a few eggs. This second type is the most common reproductive dysfunction and is detected in the majority of fish species reared in captivity. The third type of reproductive dysfunction is the inhibition of spawning only. Fish exhibiting this dysfunction undergo all phases of the reproductive cycle correctly, with oocytes going through vitellogenesis, FOM and ovulation, but spawning is blocked and the ovulated oocytes remain in the ovarian or abdominal cavity. This is, physiologically, the least serious of all reproductive dysfunctions, as only the spawning event is inhibited from the whole reproductive process, although the end consequence is similar, the absence of spontaneous spawning. This dysfunction is observed in salmonids and some flatfishes (e.g. turbot *Psetta maxima*). In these species, manual removal of the eggs (i.e., stripping) is required. If stripping is not performed, then the eggs degenerate and are reabsorbed, but in such situations they may cause the death of the female. Although stripping and artificial fertilization is a common activity in hatcheries of these species, it still represents a serious management issue, as stripping is labor intensive and must be timed precisely in order to avoid over-ripping of the eggs and reduction in quality. It also requires repeated manipulation of the broodstock, as normally females are unshychronized and each breeder has to be checked to determine its ovulated stage, which is also stressful to the broodstock.

Although reproductive disorders are more common and serious in females, male fish also display some important problems. These are usually less important than in females and except in rare cases, males of all fish species usually are spermiating in captivity. The reproductive dysfunctions detected in captive male fish are diminished sperm volume and diminished milt fluidity, which can affect negatively the success of egg fertilization. Diminished sperm production in male broodstock represents a serious problem for those species in which hatchery production is based on artificial fertilization and the acquisition of gametes by manual stripping. The difficulties of obtaining enough sperm from male breeders can block fertilization programs and requires the handling of a higher number of male breeders. On the other hand, for species that spawn spontaneously in the tank, the production of highly viscous milt reduces the rapid dispersal of the spermatozoa and thus reduces the sperm fertilization capacity.

6. TECHNIQUES FOR ENVIRONMENTAL MANIPULATION OF FISH REPRODUCTION

As described above in the environmental section and more fully in the reproductive dysfunction section, when environmental conditions are not optimal, as is often the case for fish held in captivity, maturation may be arrested until conditions are experienced that allows maturation to proceed or under extreme conditions reproduction may be aborted and the gonads regressed. The aim of environmental manipulations is to provide conditions that are sufficiently close to optimal or natural conditions to induce the majority of the fish to complete maturation and spawning. Environmental manipulations can be used to achieve two purposes, 1) adjust aspects of the naturally changing captive environment to ensure that fish proceed to maturation and spawning during the natural spawning season or, 2) to provide the complete cycle of environmental changes required to induce the full cycle of maturation outside of the natural spawning season and thereby obtain out-of-season egg production. During these manipulations the primary concern is to provide the correct or optimal conditions for spawning and close to optimal conditions for gametogenesis. Gametogenesis appears to be more flexible or have a wider optimal range for development, while spawning often requires quiet precise conditions. Few studies have actually examined this environmental flexibility of gametogenesis, but a number of studies addressed environmental effects on spawning; out-of-season spawns have been obtained by using photoperiod manipulation also in avertedly altered the timing of gametogenesis to coincide with quiet extreme temperatures compared to temperatures normally experienced during gametogenesis and apparently with no adverse effect on spawning or egg quality in rainbow tout (*Oncorhynchus mykiss*) [42,141], Atlantic salmon (*Salmo salar*) [142] and European seabass (*Dicentrarchus labrax*) [43].

To ensure spawning during the natural spawning season the primary considerations are tank environment, water quality, space and social aspects and temperature (assuming that photoperiod is ambient). Simply put water quality should be the best available, sex ratios need to be correct of the spawning species, stocking densities should be low (*e.g.*, <5 kg m^{-3}) and sufficient space is required for the formation of social groups and courtship. Lastly and perhaps most importantly, the temperature should be in the range that the species requires for spawning. Examples described below in the food species section include selection and transfer of the correct proportion of male and female adult carp and catfish to specially prepared spawning ponds which provide the space, spawning substrate and the required temperature profile

and selection of Atlantic salmon broodstock and transfer to fresh water facilities with naturally decreasing temperatures. These kinds of manipulations can also be used to alter the timing or increase the length of the spawning period, advancing, delaying and extending the window of optimal temperatures has been observed to advance, delay or extend the spawning period (see below catfish).

To obtain out-of-season spawning, both photoperiod and temperature should be manipulated. In the rainbow trout it has been established that it is the change in photoperiod or daylength, rather than actual daylength that entrains rhythms controlling maturation [41-42]. Therefore, as maturational development in the rainbow trout is entrained by the spring-summer-autumn photoperiod the application of such a photoperiod outside of the normal seasons will alter the timing of maturation and spawning [41-42] and it has been demonstrated that a phase shifted photoperiod (displacing the entire 12 month photoperiod by a number of months) will phase shift maturation, a compressed photoperiod (12 month cycle compressed to less than 12 months) will advance maturation and an expanded photoperiod (12 month cycle expanded to more than 12 months) will delay maturation. It has also been demonstrated that square wave photoperiods (direct increases or decreases in daylength) can have a similar effect as gradually changing photoperiods that resemble a natural photoperiod. However, a number of aspects need to be considered. First, spawning may not coincide in relation to the altered photoperiod as was observed under natural conditions, for example with a compressed photoperiod spawning can be expected to be later in relation to the compressed photoperiod (but earlier compared to controls under ambient conditions), i.e. winter or even spring spawning rather than autumn for trout [42]. Second, when apply a modified photoperiod to fish under ambient conditions any change made to start the photoperiod will affect the endogenous rhythms, for example applying an increasing spring photoperiod (increasing from LD 12:12) in the summer (LD 18:6) would be the equivalent of applying a direct decrease (autumn) followed by a short winter photoperiod and finally an increasing spring photoperiod. As mentioned previously, much of the work with trout was completed under constant temperature conditions of 8-11ºC, which are within the range of optimal temperatures for rainbow trout gametogenesis and spawning. Out-of-season spawning of other species can be achieved by adjusting the photoperiod and temperature cycle in the same way that photoperiod was used to manipulate spawning for the rainbow trout. The simplest and most secure way to manipulate spawning is to use phase shifted photothermal cycles, described for gilthead seabream [31]. As understanding increases of a species response to environmental control, compressed or square wave photoperiods can be used, as described for rainbow trout [41-41], European seabass [43] and Sciaenidaes [44].

Lastly a few principally tropical or sub-tropical species appear not to require periods of gonadal regression, resting and recrudescence, which seasonal environmental cycles often stimulate (in addition to maturation). Species such as tilapia, carp and red drum when brought to spawning condition through environmental manipulation and held under constant optimal spawning conditions (photoperiod and temperature) have been observed to spawn for an entire year. In the case of red drum two females and two males were maintained spawning for 7 years under a constant photoperiod of LD 12:12 and temperatures close to 24ºC [44].

7. TECHNIQUES FOR HORMONAL STIMULATION OF FISH REPRODUCTION

The development and application of hormonal therapies for the treatment of reproductive disorders in cultured fish have permitted the reproduction in captivity of several fish species that did not do so spontaneously. Hormonal therapies have not only permitted reproduction, but also allowed the improvement of the reproductive performance of broodstock and the development of a technological and economically successful aquaculture industry for several fish species [64,139,143]. In captive females exhibiting inhibition of early gonadal development (dysfunction type I), hormonal treatments can stimulate gametogenesis and oocyte maturation, but this is under investigation and not feasible yet for egg production on a commercial scale. In the case of females with inhibition of spawning (dysfunction type III), hormonal treatments are not really needed to obtain eggs, as they can be stripped, but it is often used as a management tool to synchronize ovulation and thus accelerate egg acquisition activities. It is in females with inhibition of FOM (dysfunction type II) that the use of hormonal therapies has given the best results.

There are two principal situations in aquaculture for the use of hormonal stimulation for obtaining gametes, 1) to stimulate spawning in fish species that due to reproductive dysfunction do not complete maturation in captivity or spawning is unpredictable and, 2) to synchronize spawning of the broodstock and hence improve management. Naturally, the timing of spawning of individuals in a group of broodstock held under the same conditions will approximate that of a normal distribution. The majority of the broodstock spawn during the peak of the season with some early and some late spawners. This can mean that at the beginning and the end of the spawning period sufficient eggs are not obtained to form a cohort of juveniles to stock ongrowing facilities, and, conversely, in the peak of the spawning season too many eggs may be produced for the facilities available for incubation and larval rearing. This can be a particular problem in species that spawn once a year, such as trout and salmon. Hormone stimulation can be used to synchronize and advance spawning of a group of broodfish, giving the opportunity to manage a number of large batches of eggs over the spawning season.

The history of the hormone therapies for the treatment of reproductive dysfunctions in fish has been closely linked to research discoveries in the field of reproductive endocrinology and technical advances in the purification and study of reproductive hormones. A key discovery in the area was the determination of the major endocrine failure underlying the blockage of the reproductive process in captive fish. It was demonstrated in several fishes, that the inhibition of spontaneous spawning in captivity was clearly related to the inhibition of LH release from the pituitary [31,144]. For the species under investigation, it was shown that wild fish spawning in their natural spawning grounds exhibited high levels of LH in the bloodstream during oocyte maturation, with the typical LH surge preceding ovulation and spawning [145]. In contrast, congeners held in captivity that did not spawn spontaneously, exhibited highly reduced or absent LH levels in the blood without any LH surge, even in individuals that presented high concentrations of LH in the pituitary. Thus, the blockage of ovulation was specifically related to an inhibition of LH release from the pituitary, independently of the pituitary concentration of the hormone. This discovery was further corroborated by the application of hormonal treatments that simply induced the release of the LH stored in the pituitary of these fishes, which further stimulated the progression of FOM and spawning.

The hormonal therapies developed and applied for fish aquaculture can be grouped in two major types, “first generation” and “second generation” techniques [64,139]. The first generation are the pituitary hormone based preparations, and include

the pituitary extracts and purified GTHs. The second generation are the brain hormone based treatments and includes the GnRH agonists (GnRHa) and dopamine (DA) antagonists. These two types of hormonal therapies act at different levels of the reproductive BPG axis. Drugs pertaining to the first type act directly on the gonads, while drugs of the second type act on the pituitary and thus indirectly on the gonad, through stimulation of endogenous pituitary GTH release. This is an important consideration when deciding the hormonal treatment to be applied for a specific species; the efficacy of the second generation drugs depends on the responsiveness of the pituitary of the treated fish. This means that these treatments can be totally ineffective in species in which the pituitary gland, for any reason, will not release GTHs under GnRHa treatment and thus, no effect on the gonad will be obtained. On the other hand, since first generation drugs act directly on the gonad, their efficacy does not depend on the functioning of the pituitary of the treated fish.

7.1. First generation: Gonadotropin preparations

The term "GTH preparations" refers to all those hormone preparations that display GTH activity and include pituitary homogenates, pituitary extracts (PE) and purified GTHs. These are generally named the first generation because they were the first type of hormonal treatments developed and applied for the stimulation of reproduction. They all have in common their target organ, as they all act directly on the gonad of the treated fish, to stimulate gonad development and spawning. These different preparations differ in the degree of purity of the active component (GTH) and they have been developed, historically, in relation to technological advances in protein purification.

The first gonadotropin preparations used for spawning induction of captive fish were the fish pituitary homogenates. The basis of this treatment (hypophysation) is simple and consists of the extraction of the pituitary gland from a fully mature fish, its homogenization in an appropriate buffer solution and the administration of the obtained homogenate in a recipient fish [146-147]. The homogenate induces ovulation and spawning in the treated fish. In China, hypophysation has been used extensively in the stimulation of carp reproduction, much before the understanding of the physiological bases of the treatment [139]. It was later understood that the effectiveness of the treatment is due to the high LH content of the pituitary of the donor fish. Although primitive, the application of pituitary homogenates represented for many years the only method that permitted the stimulation of reproduction in captive fishes and set up the basis for further and more sophisticated hormonal treatments. The hypophysation technique has several advantages. It is a custom made preparation that can be easily obtained in the fish farm and does not require specialized people and instrumentation for its preparation. The major disadvantage is the inaccuracy of the method. A pituitary homogenate is not a calibrated preparation and it is not known exactly the dose of the active component (LH), because of the variable LH pituitary content of the donor fish. This makes it difficult to establish an accurate method. Another disadvantage is the risk of transmission of pathogens, when transferring biological material (pituitaries) from one fish to another. This is obviously a primitive technique, maybe appropriate for small fish farms localized in remote areas, but not for the development of an intensive aquaculture facility, though the method is still employed today.

An improvement of the hypophysation method was the use of pituitary extracts, which are enriched preparations of the hormone component of the pituitary homogenate without the cellular parts. Pituitary extracts require some technical expertise and

specialized equipment and are, thus, prepared by qualified personnel. The physiological basis of the treatment is similar to the pituitary homogenates. A variety of pituitary extracts have been used in aquaculture, and some of them, such as the salmon (sPE) and carp (cPE) pituitary extracts, are available commercially. The pituitary extracts are more effective than the pituitary homogenates as they are usually calibrated using bioassays. Nevertheless, they maintain the disadvantages of risk of pathogen transmissions and high degree of species specificity [138,148-149].

The last and more sophisticated type of GTH preparations are the purified or recombinant GTHs. They require a more elaborated preparation, as only the GTH of the pituitary homogenate is used. These treatments became available with developments in GTH research and technological advances in protein purification, which allowed isolation of highly purified GTHs from a variety of species and biological sources. Purified GTHs obtained from human and mammalian biological material have been extensively used for clinical and veterinary uses, such as FSH and LH isolated from mammalian pituitaries, pregnant mare serum gonadotropin (PMSG) or human chorionic gonadotropin (hCG), isolated from the urine of pregnant women. The more common GTHs used for spawning induction of fish are hCG and purified fish GTHs. Functionally, the hCG display LH bioactivity, as it binds to gonadal LH receptors and stimulate ovarian and testicular development, gamete maturation and gamete release. The hCG, although from human origin, has been used extensively in aquaculture, because of its high availability in the market, low cost and standardized activity. In contrast, the technological difficulties in isolating fish GTHs and smaller aquaculture market compared to human and veterinary applications, have limited the use of fish GTHs, which would be physiologically more appropriate for fish than hCG. Currently, only salmon and carp GTH preparations are available in the market for aquaculture. The use of purified GTHs has important advantages over pituitary homogenates and pituitary extracts, mostly the calibration of the preparation, which allows accurate dosing, the repetitiveness of the treatment and reduced risk of pathogen transmission of the highly purified preparations. The treatment with hCG has an important disadvantage, which relates to the complex structure of the molecule. The hCG is a large and species-specific protein, which may cause immune response when administered to non-mammalian species [139]. Such immune response in the treated fish may render them unresponsive or less responsive to successive treatments. Obviously, the best approach would be the use of purified LH from the same species to which the treatment will be applied, which is the case of spawning induction of carp using purified carp GTH (cGTH). However, this is in most cases not feasible, resulting in the use of hCG as a general GTH treatment in many farmed fish.

7.2. Second generation: GnRH agonists (GnRHa)

The second generation of hormonal therapies was developed after the discovery of the brain hormone responsible for the regulation of pituitary GTH secretion, the GnRH [78]. The application of GnRH-based therapies has important advantages over the previous GTH preparations, due to the possibility of acting at a higher level of the reproductive axis and thus promoting a more general and physiological stimulation of the whole reproductive process.

As seen in previous sections, there are several native GnRHs produced in the brain of vertebrate species, all having GTH releasing activity. The GnRH is a short decapeptide synthesized in specific areas of the brain, transported through neuronal fibers to the pituitary and released by the terminal nerves in close proximity to the

gonadotrops, were it acts immediately on GnRH–R stimulating GTH release. This role is correlated with a very short half-life in circulation. The GnRHs are degraded rapidly by specific proteolytic enzymes in circulation [150-151], making exogenous administration of native GnRHs an ineffective treatment. Studies on the structure-activity relation of native GnRHs have prompted the development of GnRHa´s, which are structurally modified GnRHs exhibiting increased GTH releasing potency when administered exogenously. The most important characteristics for a GnRHa are (a) high resistance to enzymatic degradation, (b) high binding to the GnRH-R and (c) high activation of the gonadotropes, which together determine the agonist's GTH releasing potency. Accordingly, structure modifications are mostly focused on protecting amino acids at positions 6 and 9, which are the regions of enzymatic recognition and degradation, and enhancing amino acids of the NH_2- (pGlu-His-Trp-Ser) and COOH-(Pro-GlyNH_2) terminus, which are the regions responsible for receptor binding. The native GnRHs have a half-life in circulation of ~5 min whereas the most active GnRHa´s have a half-life of around ~20 min [152]. Therefore, an injection of even the most potent GnRHa stimulates a GTH elevation in circulation for 24-72 h, although this is highly variable depending on the species and temperature [153-155].

Administration of GnRHa induces release of LH from the pituitary, which in turn stimulates gonadal maturation. In females, GnRHa treatment induces FOM, ovulation and spawning, and in males increases sperm volume and, sometimes, spermatozoa density. The GnRHa-based hormonal therapies have important advantages over GTH preparations. The GnRHa´s are easy to prepare and result in a lower cost preparation than a purified GTH. They are generic and thus useful for a wide range of species, from fish to mammals, making them widely available commercially. The small size of the GnRHa does not induce immune response in treated animals and thus repeated treatments can be applied with no desensitization problems. Also, GnRHa´s act on the pituitary inducing the endogenous release of the fish own GTHs and a more appropriate gonadal stimulation than obtained with the administration of high levels of exogenous GTHs. These advantages have made the use of GnRHa the best choice for spawning induction in fish.

The GnRHa-based treatments have a limitation, which is the short half-life of the decapeptides. The classical mode of administration is the intra-peritoneal or intra-muscular injection of saline-dissolved GnRHa, at the required dose. Depending on the GnRHa type, fish species and water temperature, a single GnRHa injection induces an LH surge that lasts around 12-72 hours, before the effect disappears. In some cases, this short-lived effect of a single GnRHa injection is enough to induce spawning 2-3 days after treatment. But, in many cases, further injections are necessary to induce prolonged LH release and stimulate complete gonad maturation and spawning. In females, multiple GnRHa injections are normally required for asynchronous or multiple-batch group synchronous species, and also in species whose gonadal development is inhibited at early stages and need prolonged stimulation to affect the whole maturation process. In males, multiple injections are normally recommended, because of the asynchronous type of development of the testes. However, multiple injections are hazardous and stressful to broodstock, and in the long term can cause inhibition of reproduction by itself, appearance of stress-associated pathologies and even the death of some breeders.

For these reason, GnRHa treatments have been also administered in the form of sustained-release delivery systems [64-65]. The single administration of GnRHa via delivery systems causes sustained delivery of GnRHa to the bloodstream and thus a prolonged stimulation of pituitary LH release, which can last for several weeks, depending on the species and temperature [156-157]. A single administration of a

delivery system replaces efficiently the effect caused by 4-5 injections. Many different types of delivery systems have been developed for GnRHa administration, including pellets of cholesterol-cellulose, Ehtylene-Vinyl Acetate (EVAc), biodegradable microspheres, osmotic pumps, etc. One of the most convenient and efficient preparation are the implants of EVAc, which can be easily applied into the dorsal musculature with a syringe type applicator [140,159-160]. These implants have been proved to induce ovulation, spermiation and spawning in a wide range of fish species, being also highly efficient in the stimulation of male spermiation.

Other than GnRHa, there is another type of second generation hormone treatments, the dopamine antagonists (DAant), which are drugs that block the dopamine (DA) system of the brain, something necessary for the stimulation of reproduction of several fish species. As described previously, the endocrine regulation of reproduction is under a dual control from the brain, the stimulatory action of GnRH and the inhibitory action of DA. The activity rate of both systems determines finally the endogenous stimulation or inhibition of LH release. Not all fish species posses an active DA system in the brain. It seems that the DA inhibitory system is strong in fresh water species, but weak or absent in most marine species. The activity of the DA inhibitory tone also varies depending on the season and physiological steroid levels of the fish. In fish species with an active DA system, the inhibition of ovulation and spermiation in captivity is caused by both, increased DA activity and decreased GnRH activity, whereas in fish species with a weak DA system, reproductive disorders are due almost exclusively by decreased GnRH activity. A strong DA inhibitory tone has been demonstrated in cyprinids, silurids, salmonids and some perciformes. In these species, treatment with GnRHa only stimulates LH release, similarly to the treatment with only DAant, but is the combined treatment (GnRHa + DAant) which provides best results. Normally, only the co-treatment is efficient in the stimulation of oocyte maturation, spermiation and spawning. There are several DAant´s available in the market that proved to be useful for hormone treatments in aquaculture, the most common being pimozide, domperidone and metoclopramide. Normally, they are administered as a liquid solution injected intraperitoneally or intramuscularly, on a weekly bases.

8. GENERAL PROTOCOL FOR SPAWNING INDUCTION AND STRIPPING

The basis for developing a protocol to control reproductive maturation in a fish species is the understanding of its reproductive strategy and reproductive cycle in relation to environmental conditions, particularly photoperiod and temperature. Knowledge of sexual differentiation, size at first maturity, maturational development in relation to environmental changes, reproductive endocrinology, spawning behaviour and egg parameters, enable the design of a protocol for maintaining the fish in conditions that allow maturation to advance to late stages of gametogenesis or spawning (Table 2). The protocol describes the nutrition, environmental conditions, light, photoperiod, temperature, space, substrate and social conditions required during maturation and, when necessary, the hormonal therapy required to stimulate final maturation. Generally, for a species with a long history in aquaculture such information is known under captive conditions and protocols, which may not have actually been described, are routinely followed and not necessarily fully understood. Where a protocol is followed but not fully understood it can be difficult to explain an unexpected change in reproductive development caused by, for example, abnormal environmental conditions, whereas a full understanding of a protocol, and hence the conditions required, may enable anticipation of possible problems.

Table 2: Reproductive information of interest for designing a spawning protocol and how the information may be used in practice.

For a species with no established aquaculture history, the following sources of information can be used to propose protocols that have good opportunities for success:

- Studies on reproductive strategies, maturational development and egg parameters from wild populations,
- Anecdotal information from fishermen, whom often have a very sound knowledge of the seasonal changes in a species' gonad,
- Information on environmental parameters from areas where mature individuals of the particular species are caught, and
- Extrapolation from reproductive strategies, maturational development and egg parameters from as many closely related species as possible.

In particular, the protocol should identify critical points, such as broodfish selection, nutrition before and during vitellogenesis, environmental conditions during vitellogenesis and spawning, space and social conditions for spawning and hormone therapies required. The most common critical points that cause problems with maturational development and, therefore, the definition of a protocol to obtain gametes can be ordered as nutrition > environmental factors that control or entrain the initiation and progress of vitellogenesis or spermatogenesis > environmental factors that control or entrain final maturation and ovulation or spermiation > hormone induction therapies (Fig. 7). Nutrition has implications on both the decision to proceed with maturation in a given year (based on energy reserves and energetics, see environment section) and whether all the necessary nutritional components are available for the developing gametes. When an adequate nutrition is not provided, profound effects have been observed on fecundity and egg quality. Amongst the most important nutritional aspects for reproduction are protein quality, lipid/fatty acid composition, and vitamins. Often the only solution to poor spawning due to inadequate nutrition is to improve the nutrition for the next maturational episode [31]. No environmental or hormonal control will solve a nutritionally-based problem, and before any attempts are made to environmentally or hormonally control maturation it is essential that the broodstock nutrition is adequate (Fig. 7).

Figure 7: Flow diagram of critical points in the induction of spawning in captivity.

Incorrect or sub-optimal environmental parameters during gametogenesis and/or final maturation will result in respective primary or secondary reproductive dysfunctions (see reproductive dysfunctions). Solutions include improving the captive environment or applying hormonal therapies, when it is technically not possible to improve conditions or the optimal conditions are not known. Primary dysfunctions require a prolonged hormone therapy to stimulate the entire gametogenic period, similar to that used to spawn the freshwater eels (see protocol below). Secondary dysfunctions, which are the most common dysfunctions encountered in aquaculture, require a short-term hormone therapy to induce final maturation, ovulation or spermiation. This type of therapies have been used in various fishes, including carps, basses, bream, drums, croakers, grouper, snapper, salmonids, catfish, mullet, flatfish and puffer fish. When

developing a hormone therapy for a secondary dysfunction, two critical factors should be considered, 1) the stage of ovarian development, often measured as oocyte size, and 2) the hormone dose. Generally, spawning with hormone induction therapies is compromised when fish are treated outside of an optimal range of oocyte size or hormone doses [161]. Fish with smaller than the minimum oocyte diameter either do not spawn or exhibit a poor spawning response [161-164]. Higher than optimum doses result in reduced egg quality [161,165] and lower doses result in reduced spawning frequency [161,165-166]. There appears to be a relationship between minimum oocyte size for successful induced spawning and egg size (Fig. 8) and the equation, Minimum oocyte size = -95.98 + 0.624 x Egg size, can be used to give an indication or starting to identify the required oocyte size for developing a hormone induction therapy.

Figure 8: Regression of minimum oocyte size (μm) for successful hormone induced spawning against egg size (μm). Data plotted from review table 7-4 in [167].

Optimal hormone doses should be determined for each species. A starting point is to examine doses that have been used in closely related species. Some optimal doses for several fish species are presented in table 3. It should also be considered that oocyte size and hormone dose appear to interact [161].

Table 3: Selected hormonal therapies that have successfully induced ovulation or spawning in a range of species from different families. Similar therapies have been grouped as an indication that the therapies could be considered for related species. Inj. = injection, imp. = implant, PIM = pimozide, MET = metaclopramide.

When ovulation has been achieved but the gametes must be stripped, a final consideration is the timing of stripping in relation to ovulation. After ovulation, unfertilized eggs left in the ovarian or abdominal cavity have been observed to over-ripe, a process by which the eggs lose viability, as measured by percentage of fertilization and embryo development [39]. Over-ripening appears to be a process in which the eggs undergo a series of morphological and chemical changes, which are either the result of or the cause of the eggs losing viability. A number of species undergo a ripening period before over-ripening begins, i.e. after ovulation the viability of the unfertilized eggs may increase before decreasing due to over-ripping, resulting in an optimum period for stripping and fertilization. Care should be taken to strip eggs during the period of optimal viability [39,167].

Maturation in fish is a very complex process and its manipulation and achievement of the spawning of good quality eggs requires a complete understanding and careful consideration of all the different factors that influence the outcome of the manipulations. Many studies have demonstrated that fish can be environmentally and/or hormonally manipulated to spawn good quality eggs, equal in quality to eggs from naturally spawning fish [31,39,42]. It should, therefore, be considered that poor egg quality from manipulated fish indicates that some aspect of the spawning protocol was not correct and all parameters should be reconsidered.

9. ESTABLISHED PROTOCOLS FOR CULTURED FISH

As mentioned in the introduction, the production of finfish from aquaculture is the fastest growing agriculture industry in the world; our understanding and control of reproduction forms the basis for this growing production. In this section, the knowledge on spawning protocols for a selected group of important species is compiled and summarized. Summaries have been focused on the top seven (by volume of production) orders of fish that are being cultured, that is the Cypriniformes, Perciformes, Salmoniformes, Gonorynchiformes, Siluriformes, Anguilliformes and Mugiliformes [6] and a further three orders, Tetradontiformes Acipenseriformes and Pleuronectiformes, which are relatively important in terms of production or as a priority species for aquaculture diversification. For Perciformes, due to the diversity of families and the presence of many promising aquaculture candidates, species from ten different fish families or "groups" are described, such as tilapias, basses, snakeheads, breams, amberjacks, drums, groupers, cobia, tunas and snapper. This information can be consulted as a guide to how spawning has been achieved for the species described and as an indication of the problems and solutions that may be encountered when working with either an established aquaculture species or a candidate aquaculture species that is related to the described species.

9.1. Cypriniformes - Carps (Cyprinidae)

Generally, in captivity carps will reach late stages of vitellogenesis and spermiation, and can either be hormonally induced to spawn or left to spawn naturally in specially prepared ponds or natural waterbodies. Dopamine inhibition of GnRH action has been demonstrated. In 2005, species from the family Cyprinidae had the highest aquaculture production (9,428,518 T) of any taxonomic family [6]. The highest producing countries were China (4,999,307 T) and India (2,558,599 T). The dominant species being produced were 3,043,712 T of common carp (*Cyprinus carpio*), 2,086,311 T of crucian carp (*Carassius carassius*), of the Chinese carps were 3,904,799 T of grass carp (*Ctenopharyngodon idellus*), 4,152,506 T of silver carp (*Hypophthalmichthys molitrix*) and 2,208,678 T of bighead carp (*Hypophthalmichthys nobilis*), and of the Indian carps were 1,235,992 T of catla (*Catla catla*) and 1,195,965 T of roho labeo (*Labeo rohita*). The carps are gonochoristic and have been reported to mature in the first year, but larger 2- or 3-year-old common carp (1-5 kg) are considered the most adequate for broodstock [57]. In general, natural spawning requires warm spring or summer conditions either in ponds or flooded areas [58]. Carps are batch spawners and species such as the common carp will spawn all-year-round (5 spawns) in the tropics and just once a year in northern latitudes. Spawning is polygamous and eggs are scattered onto substrate, vegetation (common carp), river beds (Chinese carps) or flooded areas (Indian carps). Eggs or fry for aquaculture are obtained from natural spawning in natural water bodies (particularly in Asia), in specially prepared ponds (common carp) or by hormonally induced spawning in hatcheries (all carps). Common carp are both spawned naturally in ponds or hormone induced spawning with carp pituitary extract (cPE) or carp gonadogropin (cGtH). In both natural and induced spawning, the broodstock must first be environmentally induced to reach the late stages of gametogenesis. In ponds on the coastal plain of Israel, vitellogenesis begins in August-September and the peak of spawning is in May, as temperatures rise above 18°C to optimal temperatures of 22-24ºC [57,182]. The timing of spawning can be estimated by counting 1,000-1,200 (2 to 3-year-old fish) or 2,000 (>3-year-old fish) degree days from when spring temperatures rise above 15°C. Therefore, vitellogenesis takes place under a natural photoperiod (LD 10:14 - 14:10 h light) and thermal cycle (13-30°C).

Spermiating males can be encountered all year round. Broodfish are carefully selected, males by the presence of flowing milt and females by the presence of migrating germinal vesicle in 65% of the cleared oocyte sample. Selected broodfish were transferred to ponds for natural spawning (male:female ratio from 1.5:1 to 1:2) or to the hatchery for hormonal induction (male:female ratio of 1:2) [57,182]. Spawning ponds were carefully prepared [57-58,182] and pond spawning was initiated by adding vegetation or fibrous spawning mats, or by introducing broodstock to a pond with substrate for spawning. In the hatchery, female fish were induced with a priming dose of 50-100 μg kg^{-1} of cGtH in calibrated carp pituitary extract or the extract of 0.1-0.2 of a carp pituitary and 10-12 h later a resolving dose of 250-500 μg kg^{-1} cGtH, or the extract of 1-1.4 pituitaries [57,182]. Eggs were hand-stripped after a latency period of around 6-14 h that varies with temperature (240-260 degree hours). The optimum period for stripping carps was reported to be 2 h after ovulation [183]. Fecundities were 100,000-300,000 eggs kg^{-1} [57-58,182] and egg size varies from 2 to 2.5 mm, depending on the size of the female [182]. Males are given a single dose of 100-250 μg kg^{-1} cGtH, or the extract of 0.5-0.8 pituitaries. Rothbard and Yaron [57] compared the two spawning methods; induction with pituitary extract can produce 1 million fry using 15 breeders (10 females and 5 males) and highly technical knowledge and infrastructure, while pond spawning can produce 1 million fry using 400-500 breeders (200 females and 200-300 males) and low technical knowledge and infrastructure. Recently, interest has focused on using GnRHa to induce spawning in carps. Alone, GnRHa will not induce ovulation in carps; it should be co-administered with a dopamine antagonist to successfully induce ovulation [62]. Several GnRHa´s have been used with the dopamine antagonists pimozide, metoclopramide or haloperidol to successfully induce ovulation [148-149,181]. However, care should be taken as different strains of common carp gave slightly different results, particularly in the synchronization of spawning [149]. Mikolajczyk et al. [181] obtained 95% ovulation of eggs with 49-54% hatching after administering 20 μg kg^{-1} of GnRHa in conjunction with 5 μg kg^{-1} of pimozide, in two doses that were administered as a priming dose of 10% followed 6-12 h later by a resolving dose of 90%. The latency time from the resolving injection to spawning was 9-13 h.

9.2. Perciformes

9.2.1. Tilapias (Cichlidae)

In captivity, tilapias complete maturation and spawn naturally. In 2005, species from the family Cichlidae had an aquaculture production of 2,025,560 T of which 1,703,125 T were the Nile tilapia (*Oreochromis niloticus*) and 43,369 T were the Mozambique tilapia (*Oreochromis mossambicus*) [6]. Most of the production originated from China (978,135 T), Egypt (217,019 T), Indonesia (189,570 T), Thailand (109,742 T), Taiwan (83,435 t) and Brazil (67,851 T). Nile tilapia is gonochoristic and mature in the first year at the earliest opportunity (>40g). The most adequate broodstock for aquaculture are 1+ year-old and a body weight of 150-250 g, as relative fecundities, spawning frequencies and larval survival were higher compared to younger or older fish [184-185]. The Nile tilapia reproduces in almost any container, and have been spawned all year round [184]. Optimal temperatures for spawning were 25-30ºC, whereas outside of this range spawning frequency decreases and spawning stops below 20ºC [186]. Vitellogenesis had a duration of approximately 14-30 days, to give monthly spawns of batches of eggs [184,187]. Dominant male broodstock defend an

area or nest to which females visit to spawn, but spawning is polygamous [184]. Broodstock groups can be biased to females in ratios of 2:1. Eggs are 2.4 mm in diameter, demersal and are incubated orally by the females. Fecundities are approximately 2,000 eggs per fish [184] or 3,000-8,000 eggs kg^{-1} [188]. A major problem for tilapia culture is the low fecundity and asynchronous spawning of broodstock groups. MacIntosh and Little [184] compared different management practices to obtain large batches of eggs for stocking facilities. For research purposes mature tilapias have been strip-spawned to obtain gametes, females are maintained separately and observed for signs of ovulation (swollen belly and genital papilla) before being stripped [189]. Nile tilapia is at an optimum for stripping 1 h after ovulation.

9.2.2. Basses (Percichthyidae, Moronidae, Centropomidae,)

Basses of the families Percichthyidae, Moronidae and Centropomidae will reach late stages of vitellogenesis and spermiation, and some will spawn naturally in captivity. However, spawning frequency can be low and unpredictable, in which case hormone induced spawning may been applied. In 2005, species from these families had an aquaculture production of 562,269 T, of which 427,487 T were from the family Percichthyidae, 100,769 T from Moronidae and 34,545 t from Centropomidae [6]. The producing countries were China with 424,857 T, Turkey with 37,490 T and Greece with 30,959 T. The dominant species being produced were 251,770 T of Japanese seabass (*Lateolabrax japonicus*), 175,687 T of mandarin fish (*Siniperca chuatis*), 95,040 T of European seabass (*Dicentrarchus labrax*), 30,970 T of barramundi (*Lates calcarifer*) and 5,729 T of hybrid stripped bass (cross between *Morone chrysops* and *M. saxatilis*). European seabass is gonochoristic and males mature normally at their second year (approx. 300 g), while females mature the following year (>500 g) [43]. Recommended size for broodstock are males of 700 g (3-4 years) and females of 1.5-2 kg (6-8 years) [190]. Vitellogenesis begins in September-October, around 4 months before spawning, which takes place from January to March [17,43]. Therefore, environmental conditions for vitellogenesis are a declining photoperiod (LD 12:12 to LD 9:15, in Spain) and thermal cycle (25-13ºC), and environmental conditions for spawning are an increasing photoperiod (LD 9:15 to 12:12) and thermocycle (10-15ºC). Spawning has been observed over the temperature range of 9-18ºC, while optimal temperatures are 13-15ºC and spawning stops at temperatures above 18ºC [190]. An influence of the temperature on gametogenesis has also been described in photothermal manipulated sea bass broodstock, showing that a reduction of water temperature, from the highest summer to mild autumn temperatures, at least 1-2 months before the spawning period is necessary for initiation of vitellogenesis and further successful egg spawning [17]. European seabass are group-synchronous batch spawners that spawn several batches of eggs over the spawning season [43,191]. Through hormonal induction, 2-4 spawns have been detected in each female breeder, during the spawning season [168,192]. Fecundities in the wild have been estimated at 2 million per fish over the spawning season [191], and in captivity 300,000-600,000 eggs kg^{-1} have been reported [17,168,190]. Spawning behaviour is polygamous and the spawned eggs are pelagic with a diameter of 1.15-1.2 mm. European seabass spawn naturally in captivity, but hormonal induction is often used to synchronize or ensure spawns, when required. Carrillo et al. [43] reviewed the development of induced spawning protocols and concluded that the most adequate protocol was the administration of two injections of 5 and 10 μg kg^{-1} of GnRHa 6 h apart, to females that had oocyte diameters > 650 μm. A latency period of 72 h from the first injection was observed before spawning, although this can vary slightly

depending on water temperature and the time of the day when the treatment is applied [193-194]. Multiple spawnings (2-4 during the season) have been obtained using repeated GnRHa injections of 10 μg kg^{-1}, that were spaced 7-14 days apart [168]. Different GnRHa delivery systems have been tested in the European seabass, including fast and slow release implants, liquid solution of microspheric implants and saline dissolved injections. These studies have shown that administration of GnRHa via sustained release delivery systems does not improve spawning performance of females, compared to the classical saline dissolved GnRHa injection [192], but increase significantly sperm production in males [158]. Environmental manipulations have been used to obtain all-year-round spawning [17,43,190]. Displaced spawning periods were obtained by altering both the photoperiod and the termal cycle, either by phase shifting the cycles, using square wave cycles or using compressed or expanded cycles. One or two month square wave period of long daylength (LD 15:9) in an otherwise constant short daylength (LD 9:15) regime could be applied each month from March through to September to obtain spawning from October through to May [43].

9.2.3. Snakeheads (Channidae)

Snakeheads complete maturation and spawn naturally in captivity, however, much of the aquaculture production is from juveniles collected from natural sources or culture ponds. In 2005, species from the family Channidae had an aquaculture production of 308,938 T of which 277,763 T were snakehead (*Channa argus*), 13,036 T Indonesian snakehead (*Channa micropeltes*), and 8,593 T striped snakehead (*Channa striata*) [6]. Most of the production originated from China (277,511 T), Indonesia (11,525 T), India (8,213 T), Thailand (7,508 T) and Nigeria (1,333 T). The striped snakehead is gonochoristic and first maturity is at about 11 months and a size of 25-30 cm [195. The striped snakehead can spawn all year round in India, but peak spawning is observed during the rainy season, which also results in a cooler period with temperatures below 30ºC. In Southern India two peak periods of spawning coincide with two rainy seasons (June-July and November) and in Northern India one peak spawning period during April to September. Snakeheads appear to form spawning pairs, which build a nest for courtship, spawning and egg incubation. The eggs float, but are spread in a kind of film over the centre of the nest. Striped snakehead eggs are 1.15-1.46 mm in diameter and water temperatures for egg incubation range from 16 to 33ºC. Fecundities for striped snakehead were reported between few hundreds and few thousands, depending on the size of the female; for other species, fecundities range from 2,214 eggs for a 50 cm fish (*C. marulius*) to 33,873 for a 22.2 cm fish (*C. punctata*) [195]. Striped snakehead has been reported to spawn naturally in captivity just two months after being air freighted from Thailand to Hawaii [196]. The broodstock were 1-2 kg and were held at 28-32ºC; the eggs were collected from the bottom of the tank. Striped snakehead has been spawned with carp and catfish pituitary extract. A dose in the range of 40-80 mg of pituitary per female given in two injections induced spawning 11-12 h after the second injection in *C. marulius*, *C. puntatus* and *C. gachua* [195].

9.2.4. Breams (Sparidae)

Generally, in captivity, the Sparidae will reach late stages of vitellogenesis and spermiation and spawn naturally, but hormone induced spawning has also been applied successfully when required. In 2005, species from the family Sparidae had an aquaculture production of 245,217 T, of which 110,705 T were gilthead seabream

(*Sparus aurata*) and 82,083 T silver seabream (*Pagrus auratus*) [6]. Most of the production originated from Japan (76,082 T), China (44,222 T), Greece (44,124 T), Turkey (28,334 T) and Spain (15,552 T). Gilthead seabream is a protandrous hermaphrodite, with individuals maturing first as males and then as females. The change from males to females occurs from one year to the next and appears to be controlled socially when the ratio of large females to smaller males decreases [31]. All gilthead seabream mature as males in the first year (approx. 200-300 g) and can, under the correct social conditions, change to females in the second or third year (approx. >600 g). Recommended size for broodstock for a hatchery are males of 300-500 g (2-3 years) and females of 1-1.5 kg (4-6 years) [190]. The gonads, from May to September, may exhibit both morphology of testes and ovary. From September onwards vitellogenesis begins in maturing females and spermatogenesis in maturing males. Environmental conditions for vitellogenesis are a declining photoperiod (LD 12:12 to LD 9:15, in Spain) and thermal cycle (25-13ºC). The spawning season extends from January to May, under an increasing photoperiod (LD 9:15 to 14:10) and thermal cycle [31]. Spawning has been observed over the temperature range of 14-20ºC, while optimal temperatures were 15-17ºC and spawning stops at temperatures above 24ºC [190]. Gilthead seabream is a batch spawner and can spawn daily for 3-4 months; fecundities can reach 2-3 million eggs kg^{-1} over the spawning season [31]. Spawning behaviour is polygamous and the spawned eggs are pelagic with a diameter of 0.94-0.99 mm. Although hormonal induction is now not needed for seabream, well established hormonal induction protocols were necessary when the industry started, with the use of wild broodstock. Zohar et al. [31] reviewed the development of induced spawning protocols and concluded that the most adequate protocol was the use of slow release implants containing 100 µg kg^{-1} of GnRHa, in females that had oocyte diameters >530 µm. A latency period of 48-72 hours was observed before spawning began in 80% of the fish and daily spawning continued for 4 months. Often a proportion of females within a pool of broodstock do not spawn naturally, and hormone induction protocols have been applied to spawn these females [166]. Environmental control can be used to obtain all-year-round spawning [31,190]. The 12-month photoperiod and thermal cycle is phase-shifted 3, 6 and 9 months to give 4 different spawning groups (including the ambient cycle). A 3-4 month spawning period is achieved soon after the period of short winter days, December, March, June and September under respective photoperiods ambient and phase shifted 3, 6 and 9 months, to give an all-year-round production of eggs. No reduction in egg quality was observed from fish spawned with hormonal or environmental control.

9.2.5. Amberjacks (Carangidae)

In 2005 species from the family Carangidae had an aquaculture production of 178,270 T, of which 159,798 T were Japanese amberjack yellowtail (*Seriola quinqueradiata*), 2,392 T Japanese jack (*Trachurus japonicus*) and 15,534 T unidentified *Seriola spp* and *Trachurus spp* [6]. Most of the production originated from Japan (164,808 T) and China (11,973 T), but more recently kinfish (*Seriola lalandi*) has also been produced commercially in Australia [197]. In Europe, the species of interest is the greater amberjack (*Seriola dumerili*), but commercial production has been hindered by substantial problems in reproduction, as cultured fish often fail to reach advanced stages of gametogenesis [140,198-199]. Depending on the species, amberjacks reach reproductive maturation at 2 years (*Seriola rivoliana*) [200] or may require more than 4 years to mature (greater amberjack), reaching sizes of >5 kg [201-

202]. Amberjacks in the temperate zone initiate their reproductive cycle in the spring and spawn in the early summer months (June-July), at water temperatures ranging between 21 and 25ºC [140,203].

All amberjacks are either group-synchronous multiple-batch spawners or asynchronous spawners, and depending on the species, they can spawn a few times during the reproductive season, as in greater amberjack and kingfish [140,197,201,203], or can spawn on a daily basis for many weeks, as is the case in the Japanese yellowtail [204-205]. However, the response to hormonal therapies is not the same in all amberjacks. For example, in the greater amberjack hormonal treatment with either a single injection or a GDS may not always induce more than a single spawning event [206], whereas in the Japanese yellowtail, a GDS induced multiple spawning [204]. Spawning induction in amberjacks has been achieved with the use of hCG injections [205,207-208] and GnRHa GDS (40 μg kg^{-1}) [140].

Egg fecundity can be extremely variable in response to hormonal treatment, which is probably related to the stage of reproductive development at the time of hormone treatment (see earlier section). In the greater amberjack, fecundity may range between 3,000 and 43,000 eggs kg^{-1} [140,208], whereas in Japanese yellowtail it may range between 18,000 and 172,000 eggs kg^{-1} [205,207].

9.2.6. Drums, croakers (Sciaenidae)

Generally in captivity, the Sciaenidae reach late stages of vitellogenesis and spermiation and spawn naturally, however, spawning frequency can be low and unpredictable, in which case hormone induction of spawning was used. In 2005 species from the family Sciaenidae had an aquaculture production of 117,141 T, of which 69,641 T was large yellow croaker (*Larimichthys croceus*), 46,632 T red drum (*Sciaenops ocellatus*) and 800 T meagre (*Argyrosomus regius*) [6]. Most of the production originated from China (115,383 T). Red drum is gonochoristic and matures at a size of 4-5 kg or 4-5 years in wild stock, however, the age has been reduced to 2 years in cultured fish [44]. Vitellogenesis has been observed to begin in July-August indicating a period of 1-3 months before the peak of spawning in September-October [209]. Therefore, environmental conditions for vitellogenesis are decreasing photoperiod and temperatures of 28-30ºC [209]. The spawning season extends from August to January with a peak in September-October, i.e. under the declining autumn photoperiod and thermal cycle. Spawning has been observed at 24-26ºC, but stops below 20ºC [44]. The red drum was described as group synchronous batch spawners [210] and two females have been recorded to spawn 10-20 times during one month [44]. The eggs are pelagic, with a diameter of 0.9-1.0 mm and fecundities over the spawning season have been estimated at 3×10^7 eggs per 9-14 kg female [211]. Although hormonal induction is not needed for red drum, studies have indicated that the sciaenids, red drum, spotted sea trout (*Cynoscion nebulosus*), orangemouth corvine (*Cynoscion xanthulus*) and Atlantic croaker (*Micropogonias undulates*), with mean respective oocyte diameters of 600 μm, 400 μm, 440 μm and 550 μm, can be spawned with a single injection of 20-100 μg kg^{-1} of GnRHa [44].

An amazing aspect of the red drum and other sciaenids has been the environmental control of spawning using phase-shifted and compressed photoperiod and thermal cycles. Thomas et al. [44] reviewed how two females and two males were exposed to an abbreviated photoperiod and thermal cycle until the autumn spawning environment was reached and held at LD 12:12 and 24ºC. Under these conditions the fish spawned continually for seven years, producing approximately 250 million

fertilized eggs from 360 spawns. During the seven year period variations in the temperature were simulated to control the spawning, optimal spawning was obtained at 24-26ºC, spawning slowed at below 23ºC and stopped at below 20ºC. Meagre is a promising candidate for aquaculture in the Mediterranean, and females with oocyte sizes >500 μm were successfully induced to spawn with 50 μg kg^{-1} GnRHa implants (Duncan et al., unpublished).

9.2.7. Groupers (Serranidae)

In 2005, species from the family Serranidae had an aquaculture production of 65,815 T, most of which (61,815 T) was not reported by species [6]. Most of the production originated from China (38,915 T), Taiwan (13,582 T), Indonesia (6,883 T), Malaysia (2,572 T) and Thailand (2,280 T). There are various species of groupers with interest for aquaculture [212], some of them being the dusky grouper (*Epinephelus marginatus*) [212], the white grouper (*E. aeneus*) [214], the Nassau grouper (*E. striatus*) [215], the spotted grouper (*E. akaara*) [216], the honeycomb grouper (*E. merra*) [217] and the sevenband grouper (*E. Septemfasciatus*) [218]. Groupers are protogynous hermaphrodites, with sex inversion taking place at a rather large size and age [219], and in the wild a single male usually fertilizes the eggs of many females [220]. The reproductive season is in the summer, and the spawned eggs are pelagic in nature, but are smaller than other marine fishes (< 800 μm in diameter). Groupers have a group-synchronous multiple-batch ovarian development and spawn over an extended reproductive season.

Spontaneous spawning in captivity is difficult in groupers [221-222], and it usually requires the employment of very large tanks or ponds, and low stocking densities [215,223-224]. This may be due to an elaborate breeding behaviour and pairing requirement of most groupers [216,221,225-226]. In addition, the large age-size that fish undergo sex inversion results in a scarcity of males and the need to artificially sex invert females to functional male [227]. Female often do not complete vitellogenic growth and the diameter of the oocytes is too small for hormonal induction. In cases that oocytes reach the end of vitellogenesis, induction can be done using GnRHa [228] or hCG [229] in injectable form, or using GDS [159,214]. The use of GDS for the induction of ovulation in groupers is advantageous to injectable forms, as it may stimulate multiple ovulation events [159]. Still, no available system can induce spawning for the whole duration of the natural reproductive season, and re-administration of the hormonal therapy may be necessary. As a result, total fecundity of captive females after GnRHa implantation is lower than reported for wild groupers from the natural environment.

9.2.8. Cobia (Rachycentridae)

Generally in captivity, cobia complete maturation and spawn naturally. In 2005 cobia (*Rachycentron canadum*) had an aquaculture production of 22,751 T; no other species from the Rachycentridae family has a reported aquaculture production. Most of the production originated from China (18,882 T) and Taiwan (3,863 T) [6]. Cobia is a rapidly emerging aquaculture species; in 1999 world production was just 820 T from Taiwan. Cobia is gonochoristic and mature at 1-2 years old, with a size of 10 kg [230-231]. Under natural conditions in ponds in Taiwan, cobia have been spawned all-year-round with two peak spawning periods, one in the spring (February-May) and a second in the autumn (October) [230-231]. Suitable temperatures for spawning were 24-29ºC

[230], and peak spawning was at tempertaures of 24-26ºC [230] and 23-27ºC [231]. Liao et al. [231] described how cage reared mature fish were selected and 100 breeder (ratio males:females of 1:1) were transferred to each pond with an area of 400–600 m^2 and 1.5 m depth. Spawned eggs were collected from the ponds, eggs were pelagic and 1.35-1.40 mm in diameter.

9.2.9. Tunas (Scombridae)

In 2005, species from the family Scombridae had an aquaculture production of 22,995 T, of which 7,869 T was Pacific bluefin tuna (*Thunnus orientalis*), 7,583 T Atlantic bluefin tuna (*Thunnus thynnus*) and 7,458 T Southern bluefin tuna (*Thunnus maccoyii*) [6]. Most of the production originated from Mexico (7,869 T), Australia (7,458 T), Croatia (3,425 t) and Spain (3,364 T). The tuna aquaculture is a "capture-based" industry [232] and involves the capture of migrating wild fish and their fattening in floating cages, for periods ranging from 2 months to 2 years [232-236]. Tunas are asynchronous spawners with a frequency of every 1-2 days [237] and spawn during the late spring, early summer at water temperatures >23°C. Being pelagic fishes, tunas migrate great distances to reach their spawning grounds, and spawning takes place on the water surface after dusk. The eggs are positively buoyant with a diameter of about 1 mm.

Efforts at developing a captive tuna broodstock were initiated in Japan with the Pacific bluefin tuna [236,239]. Broodstock weighing >100 kg are maintained in large cages or enclosures and are allowed to spawn naturally. Fish reared from eggs obtained during the 1990's at Kinki University reached reproductive maturation in 2004 and spawned in captivity [239]. Currently, a small number of fully farmed-raised Pacific bluefin tuna are sent to the market on a regular basis. However, spawning is not consistent, since broodstock maintained in sea cages can not be exposed to the optimal thermal conditions for reproductive maturation and spawning, and it has been observed that lower temperatures may delay or abolish the spawning season in a given year [241]. On the other hand, yellowfin tuna (*Thunnus albacares*) broodstock have been maintained in land-based tanks, and have spawned naturally over many years [241-242].

Recently, a GDS-based method for the induction of spawning in captive Atlantic bluefin tuna has been developed [160]. The GnRHa implants were prepared by loading GnRHa into a matrix of poly [Ethylene-Vinyl Acetate] and the implants were attached to a p[ethylene] arrowhead using a 0.5 mm nylon monofilament. Administration of the GDS was done underwater using a spear gun fitted with a specially designed spearhead, since tunas can not be anaesthetized and treated with the necessary hormones. After GDS treatment, FOM and post-ovulatory follicles occurred in 63% and 88%, respectively, of the GnRHa implanted females, compared to 0% and 21%, respectively, of the control females. In addition, eggs were obtained from GnRHa-implanted females and were fertilized in vitro with sperm from spermiating males, which resulted in viable embryos and larvae. Finally, fertilized eggs were collected from the cages after three days from the GDS administration.

9.2.10. Snappers (Lutjanidae)

Generally in captivity, the Lutjanidaes will mature to the late stages of vitellogenesis and spermiation and different species will either spawn naturally or be hormonally induced to spawn. In 2005 species from the family Lutjanidae had an aquaculture production of 3,911 T, of which the dominant species was the mangrove red

snapper (*Lutjanus argentimaculatus*) with a production of 3,699 T. Most of the production originated from Malaysia (3,452 T) [6]. Snapper are gonochoristic and the lunarejo (*Lutjanus guttatus*), a species with good aquaculture potential from the eastern coast of the Pacific (Mexico to Peru) mature at a size of 250g from the second year (personal observation). The snapper tend to have long spawning seasons that cover most of the year, but which have peak periods. Grimes [243] observed that the peak periods were associated with peaks in the productivity of the ecosystem; generally, species in stable island environments spawned throughout the year whilst species with continental environments, with seasonal ecosystem productivity, present long spawning periods with peaks. The lunarejo exhibit the gonadal morphology of an active batch spawner during the whole year, with peak periods during March-April and August-November on the Mexican coast [244]. Vitellogenesis is short, 1-2 months, from February to April [161,244] under an increasing photoperiod (LD 11:13 to 12:12) and temperature (22 to 24°C). Spawning is polygamous, with the snapper forming spawning aggregations [243]. Lunarejo eggs are pelagic, with a diameter of 0.8 mm and scattered into the environment [161]. Fecundities were 70,000-100,000 eggs kg^{-1} for lunarejo that were induced to spawn. Many snapper species have been observed to spawn naturally in captivity, e.g. *L. campechanus* [245], *L. kasmira* [246], *L. stellatus* [247], *L. argentiventris* [248] and *L. argentimaculatus* [177]. However, other species do not spawn naturally in captivity, but hormonal induction has been used successfully, both hCG and GnRHa treatments. Lunarejo with an oocyte size >440 μm were successfully spawned with 240–280 μg kg^{-1} GnRHa implants [161]. The mangrove red snapper, *Lutjanus argentimaculatus*, the principal species being produced, have been induced to spawn with a single injection of 100 μg kg^{-1} GnRHa or a single injection of 500 IU kg^{-1} of hCG when females had an oocyte size >400 μm [177].

9.3. Salmoniformes - Salmon, trout (Salmonidae)

Generally in captivity, the Salmonidae complete maturation through to ovi-postion and the gametes must be manually stripped and fertilized. In 2005 species from the family Salmonidae had an aquaculture production of 1,950,578 T, of which 1,235,972 T were Atlantic salmon (*Salmo salar*) and 486,928 T rainbow trout (*Oncorhynchus mykiss*) [6]. Most of the production originated from Norway (641,174 T), Chile (598,251 T), Scotland (142,613 T) and Canada (103,164 T). Atlantic salmon is gonochoristic and exhibits a plasticity of maturational strategies, which results in a proportion of individuals from a cohort maturing each year. This plasticity of maturation strategies depends on environmental and genetic influences that determine when maturation can proceed to completion [37,40,249]. The most adequate broodstock for aquaculture are the late maturing genetic strains with a minimum first maturation at 2+ sea winters (3+ kg) (3+ years old, 1 year in fresh water + 2 years in sea water). Vitellogenesis last for approximately 10 months, beginning in the spring-summer months [250-251]. It is clear that the majority of the oocyte growth attributed to vitellogenesis takes place during spring to autumn under a spring-summer-autumn photoperiod (in Scotland LD 18:6 to 6:18) with appropriate thermocycle (in Scotland 2-14ºC). The spawning period is during autumn-winter. Different genetic strains and different environmental conditions due to different latitudes result in different spawning periods, but the average peak of spawning in Scotland is in November [252]. Female broodstock spawn once a year. Optimal spawning conditions in Scotland are short daylenth (6-8 h) and temperatures bellow. No ovulation and poor spermiation were observed at temperatures above 13ºC [175,253] or 16ºC [254]. GnRHa has been

successfully used to induce ovulation and spermiation in fish maintained at 14-16ºC [171]. Wild broodstock migrate from sea water to fresh water to spawn and generally culture practice is to transfer broodstock to fresh water for ovulation and stripping. A reduced proportion of females left in sea water were observed to ovulate [255]. GnRHa has been successfully used to induce ovulation in females left in sea water (personal observation). During courtship, in the natural spawning areas, the females excavates a reed (gravel nest) into which the eggs are spawned, fertilized and buried with gravel. Spawning is polygamous, a female spawns with a dominant male and precocious male sneakers. Eggs are 5-6 mm in diameter and demersal [256]. In culture, in the absence of substrate, the females will not release the eggs and gametes must be manually stripped and fertilized. In rainbow trout the optimum time to strip eggs was 4-6 days after ovulation [257]. Fecundities are approximately 2,000-4,000 eggs kg^{-1}. Hormone stimulation is not necessary to obtain ovulation or spermiation, but is a useful management tool to enhance ovulation or spermiation when conditions, such as temperature and salinity, are not correct (see above) or to synchronize and advance ovulation or spermiation. Sustained release of GnRHa using implants, microspheres or FIA-emulsion has been used to synchronize and advance ovulation in Atlantic salmon [156,175,258] and rainbow trout [259-260]. Successful doses were 20-50 μg kg^{-1} for rainbow trout [259-260] and 50 μg kg^{-1} for Atlantic salmon [175]. It was recommended to apply the sustained GnRHa delivery systems up to 6 weeks before the anticipated date of spawning, to obtain ovulation of 80-100% of the fish within 2 weeks of treatment [261-261]. Rainbow trout has been environmentally manipulated with altered photoperiods under constant temperatures to spawn all-year-round [41-42]. Although not as thoroughly studied, the Atlantic salmon appears to respond similarly to photoperiod manipulation [175,263-264]. It is important to ensure that optimal temperatures are provided during the displaced spawning period [42,175].

9.4. Gonorynchiformes - Milkfish (Chanidae)

Generally a large percentage of milkfish are cultured from wild juveniles, however, the proportion of hatchery produced juveniles is increasing (10% of production in the Philippines and the bulk of production in Indonesia) [265] and the majority of eggs for culture appear to be obtained from natural spawns in cages, ponds and tanks. In 2005 milkfish (*Chanos chanos*) had an aquaculture production of 594,783 T [6]; aquaculture production was not reported for any other species from the Chanidae family. Most of the production originated from the Philippines (289,153 T), Indonesia (254,067 T) and Taiwan (50,050 T). Milkfish are gonochoristic and mature at a size of >4 kg and 5 years, however, fish as old as 9 years are required for optimum egg quality [58]. Vitellogenesis or gonadal maturation occurs during February and March before the 6-7 month spawning season, the timing of which varies across the species geographic distribution. Temperatures of 26-29ºC and 29-30ºC were indicted for spawning and egg development [173-174,266]. Natural spawning has been achieved in cages and ponds with 1:1 sex ratios [58,266]. Spawning occurred in relation to the lunar cycle between midnight and 06.00 h and appeared to be polygamous with males chasing spawning females. The eggs were pelagic with a diameter of 1.1-1.25 mm. Fecundities have been estimated at 25×104 eggs kg^{-1}. Hormonal induction therapies have been developed, but due to difficulties related to handling large delicate broodstock research has focused on natural spawning. Marte et al. [173-174] successfully induced spawning using either hCG or GnRHa. Spawning was induced with a single injection of 1,000 IU kg^{-1} of hCG and either an injection of 10-33 μg kg^{-1} of GnRHa or an implant of 19-36 μg kg^{-1}. The

latency period between hormone administration and spawning was 16-49 h. Marte et al. [174] recommended an oocyte size of 730-780 μm for hormone induced spawning.

9.5. Siluriformes - Catfish, pangasius (Pangasiidae, Ictaluridae, Siluridae, Clariidae, Bagridae)

Generally in captivity, the catfish species will mature to the late stages of vitellogenesis and spermiation (particularly the families Ictaluridae, Siluridae, Clariidae) and can either be hormonally induced to spawn or left to spawn naturally in ponds, depending on the species. However, some species particularly in the Pangasiidae family must be hormonally induced to spawn. Dopamine inhibition of GnRH action has been demonstrated in the North African catfish (*Clarias gariepimus*) [267]. In 2005 species from the order Siluriformes had an aquaculture production of 1,468,357 T of which 440,611 T were from the family Pangasiidae, 382,112 T from Ictaluridae, 287,588 T from Siluridae, 264,723 T from Clariidae and 92,900 T from Bagridae [6]. The dominate species being produced were 440,611 T of pangas catfish (*Pangasius spp*), 379,707 T channel catfish (*Ictalurus punctatus*), 286,330 T amur catfish (*Silurus asotus*), 114,311 T of a catfish hybrid (cross between *Clarias gariepinus* and *C. macrocephalus*), 110,876 T of torpedo-shaped catfish (*Clarias spp*.), 84,565 T of yellow catfish (*Pelteobagrus fulvidraco*) and 28,746 T of North African catfish (*Clarias gariepimus*). Most of the production originated from China (478,004 T), Vietnam (376,000 T), USA (275,754 T), Thailand (130,784 T) and Indonesia (102,090 T). The channel catfish is gonochoristic and mature after 2-3 years at sizes greater than 1.5 kg, but for culture, broodstock of 3-4 years are recommended [268]. Vitellogenesis has been observed to begin in November and lasting for 6 months, before the spawning season in May-July [269-270]. Environmental conditions for vitellogenesis were a winter photoperiod and temperatures of 15-25ºC [269]. The spawning season extended from May to July under the spring photoperiod and temperatures of 24-30ºC [269] and spawning stopped at temperatures above 30ºC. The channel catfish form pairs for spawning, the pair excavates a hole or nest where the egg mass is laid and the male guards the eggs during incubation. The females spawn once a year, with a fecundity of approximately 8,000,000 eggs kg^{-1}. The eggs are 3 mm in diameter, demersal and adhesive. Under culture conditions channel catfish spawn naturally in spawning ponds, utilizing 20-40 L spawning containers from which the egg masses can be collected [268]. Broodstock are stocked at 500-1,000 kg ha^{-1}, at a ratio of 1:1 to 3:5 males to females. Although hormonal induction is not needed and little used for channel catfish, studies have shown that successful spawning was possible with a latency period of 24-72 h after injection with respective priming and resolving doses of 2 and 9 mg kg^{-1} of cPE, a single injection of 1,000-1,760 IU kg^{-1} of hCG or respective priming and resolving doses of 10 and 90 μg kg^{-1} of GnRHa [178]. Hormone induction is used for the culture of the North African catfish and successful spawning was possible after injection with either 4,000 IU kg^{-1} of hCG or 50 μg kg^{-1} of GnRHa, combined with 500 μg kg^{-1} of pimozide [178-179]. The temperature dependant latency period was 12.5 and 16 h respectively, for hCG and GnRHa at 25ºC. The inhibition of GnRH by dopamine has been described in the North African catfish, suggesting that a dopamine antagonist should be used with GnRHa [267]. The pangas catfish *spp* must be hormonally induced to spawn in captivity. *Pangasius bocourti* were observed to mature to advanced stages of vitellogenesis, but not to complete vitellogenesis. Cacot et al. [271] selected fish with a mean oocyte size of 1.1 mm and induced gametogenesis with a mean of 4 (range 1-10) daily injections of 500 IU kg^{-1} of hCG. This preparatory step induced the development

to an average ovarian content of 52% of oocytes >1.6 mm and final maturation and ovulation was induced with either a single injection of 2,000-2,500 IU kg^{-1} of hCG or respective priming and resolving does of 1,500 and 2,500 IU kg^{-1} of hCG. After ovulation, the eggs may be stripped and fertilized, in *Clarias macrocephalus* the optimum time for stripping was 10 h after ovulation [272]. Temperature has been used to both advance and delay spawning in the channel catfish. Hall et al. [269] used geothermal temperature control to heat pond water from February to April and advanced spawning by 2 months, compared to control fish at ambient temperatures. Similarly, Brauhn [273] obtained both natural and cPE induced spawns in August and November after maintaining channel catfish, from April, at 18ºC for 109 days before increasing the temperature to 26ºC over an 8 day period.

9.6. Anguilliformes - Eels (Anguilidae)

Generally eels are cultured from wild juveniles; eels do not mature in captivity. Studies have successfully induced gametogenesis and ovulation, but egg and larval quality has been poor and larval survival has not yet been sufficient for mass culture. In 2005 species from the family Anguilidae had an aquaculture production of 242,067 T of which 233,045 T were the Japanese eel (*Anguilla japonica*) and 8,329 T were the European eel (*Anguilla anguilla*) [6]. The highest producing countries were China with 179,245 T, Taiwan 28,481 T, Japan 19,744 T, Republic of Korea 5,575 T and the Netherlands 4,000 T. The life cycle of the European eel begins in the Sargasso sea where eels are believed to spawn, based on the presence of larval stages of eels, the larvae migrate with currents towards Europe and enter river systems as small juveniles [274]. In the river systems the eels grow to >40cm before leaving the rivers to migrate to the Sargasso sea. The eels prepare for migration by changing color, from yellow to silver, but at the time that the eels leave the river systems no or little gonadal development has taken place and GSI was 1-2%. The male eels leave the river systems in August with a size of 40 cm and females in September-October with a size of >50 cm. It was suggested that different swimming speeds resulted in the larger females meeting the smaller females in the spawning areas. It is not known what environment is experienced by migrating eels or what the environmental controls are for eel maturation. Despite many experiments to provide appropriate environmental stimulus for maturation, including temperature, light, salinity, pressure and swimming, little or no maturational development was observed [274]. However, both pressure (by submersion in a cage) and swimming did increase levels of maturational hormones and a slight increase in GSI. Maturational development and ovulation/spermiation have been hormonally induced in both the Japanese and European eel. The Japanese eel was first induced [275]. Males (200-300 g) were treated with weekly injections of 1 IU g^{-1} of hCG for 10-14 weeks to obtain spermiation. Females were treated with weekly injections of 20 mg $fish^{-1}$ of salmon pituitary extract (sPE) until oocytes with migrating germinal vesicle were obtained (8-13 injections). A single injection of 17,20ß-dihydroxy-4-pregnen-3-one (DHP) (2 µg g^{-1}) was given 24 h after the last injection of sPE (20 mg $fish^{-1}$) and most fish ovulated 15-18h later. Very similar protocols have been successfully applied to the European eel [276-277]. Observations indicated that the hormonally induced maturation was temperature dependant and the European eel takes about 20 days to mature at 25ºC, 60 days at 15ºC, but does not mature at temperatures below 10ºC [274].

9.7. Mugiliformes - Mullet (Mugilidae)

Generally mullet are cultured from wild juveniles. Mullet do not spawn in captivity and only a variable proportion mature to the late stages of vitellogenesis. However, studies have successfully induced spawning and demonstrated a dopamine inhibition of GnRH. In 2005, species from the family Mugilidae had an aquaculture production of 167,946 T which was almost entirely flathead grey mullet (*Mugil Cephalus*) and unidentified mullet (*Mugilidae spp*) [6]. Most of the production originated from Egypt (156,441 T), Indonesia (11,668 T) and the Republic of Korea (5,501 T). Mullet are gonochoristic with males maturing at a size 251-375 mm or 1-3 years and females at a size 291-400 mm or 2-4 years [278]. The spawning season varies considerably across the species geographic distribution; in the Atlantic coast of the USA the spawning season is October to April [279], in the Mediterranean from July to December [180] and in the Philippines peak spawning is June to August [280]. Vitellogenesis appears to take place during the 2 months before the spawning season, from August to October [279], as temperatures and photoperiod begin to decrease. Mullets migrate from brackish lagoons to spawn in full strength sea water. Spawning appears to be polygamous and the eggs are pelagic with a diameter of 0.7-0.8 mm [180]. Fecundities have been estimated at 1×10^6 to 1.6×10^6 eggs kg^{-1} [58,180]. Natural spawning has not been achieved in captivity. A range of hormonal induction therapies have been developed [281]. It was recommended to use mullet with oocyte diameters >0.6 mm. Two therapies that were 100% successful included a priming dose of 4.3-8.4 μg g^{-1} of sGtH or 14.1-21.4 IU g^{-1} of hCG and 24 h later a resolving dose of 6-16.8 μg g^{-1} sGtH or 30.4-47.6 IU g^{-1} of hCG. The latency period was 10-15 h and percentage fertilization was 87-98%. Treatments using GnRHa were not as successful; doses of 101-455 μg kg^{-1} were 68% successful with fertilization of 0-95%. However, the poor efficacy of GnRHa has been shown to be due to the inhibitory effect of dopamine [180]. Aizen et al. [180] obtained induced spawns applying a priming dose of 10 μg kg^{-1} of GnRHa combined with 15 mg kg^{-1} of metaclopramide (MET, dopamine antagonist) followed 22.5 h later with a resolving dose of 20 μg kg^{-1} of GnRHa combined with 15 mg kg^{-1} of MET. The spawning success was 83% and the latency period was 21 h. Male spermiation was stimulated with EVAc slow release implants loaded with 4 mg kg^{-1} of 17α-methyltestosterone [180]. The timing of gonadal development has been altered through environmental manipulation. Generally, short photoperiod (LD 8:16) and cool temperatures (21ºC) stimulated gonadal development and long photoperiod (LD 16:8) and high temperatures (31ºC) inhibited development [49]. A short photoperiod (LD 8:16) with either low 21ºC or high 31ºC temperatures appeared to stimulate the cortical vesicle developmental stage, long or short photoperiod with low temperatures stimulated vitellogenesis and either long or short photoperiod with high temperatures induced atresia [49,281].

9.8. Tetradontiformes - Puffer fish (Tetraodontidae)

Generally in captivity, the Tetraodontiformes will mature to the late stages of vitellogenesis and spermiation; hormonal induction is used for spawning induction. In 2005 species from the family Tetraodontidae had an aquaculture production of 24,572 none of which was reported by species [6]. Most of the production originated from China (15,407 T) and Japan (4,582 T). The botete diana (*Sphoeroides annulatus*) is a species with good aquaculture potential for the Pacific coast of America. Botete diana is gonochoristic and mature at a size of 400-500 g (3 years) (personal observation). Vitellogenesis was observed to begin in March-April, 1-2 months before spawning

[172]. Environmental conditions for vitellogenesis were a spring photoperiod (LD 12:12) and increasing temperatures (20-24ºC). The spawning season extended from April to June with a peak in May under the increasing spring photoperiod (LD 12:12 to 14:10) and thermocycle (24-28ºC). The botete appear to be group synchronous batch spawners from histology [172] and because a few individual fish in captivity have been spawned twice approx. a month apart in the same year (personal observation). Spawning appears to be polygamous and eggs are scattered into the environment. The eggs were demersal and adhesive with a diameter of 0.7 mm. Fecundities were 1×10^6 eggs kg^{-1} [172,282]. In captivity gametes should be stripped and artificially fertilized, as spawned eggs will adhere to most surfaces in the tank complicating egg collection. Few broodstock spawn naturally in captivity and hormone induction can be used to induce spawning. Duncan et al. [282] induced 82% of females to ovulate using GnRHa implants and injections, compared to 18% of control fish that spawned naturally. Females with oocyte sizes greater than 500 µm were implanted with 135±32 µg kg^{-1} GnRHa (82% spawned) or injections of a priming dose of 20 µg kg^{-1} and resolving does of 40 µg kg^{-1} (73% spawned), with a latency period of 16-40 h. Eggs were stripped and fertilized; fertilization rates were high (90-97%). Optimal time for stripping in tiger puffer (*Takifugu rubripes*) was close to ovulation (time 0), mean fertilization of eggs stripped within four h of ovulation was 70% [283]. To date, no environmental manipulation has been reported with the botete diana. However, spawning has been observed to start earlier when spring temperatures rise above 24ºC early and the season was extended when temperatures did not rise above 28ºC until late June early July (personal observation).

9.9. Acipenseriformes - Sturgeon (Acipenseridae)

Generally in captivity, the Acipenseriformes will mature to the late stages of vitellogenesis and spermiation and hormonally induced spawning has been applied. In 2005 species from the family Acipenseridae had an aquaculture production of 19,648 T, most of which was not reported by species. Most of the production originated from China (15,407 T), Russian Federation (2,470 T) and Italy (1,158 T) [6]. The sturgeons are gonochoristic and mature at a late age, the Siberian sturgeon (*Acipenser baeri*) matures after 10 years when the females reach a size of 7.9 kg and the males 5.3 kg [176]. The sturgeons are migratory fish which go to sea and return to the rivers to spawn. Spawning can be every year, every second year or even every third year and the most common strategy for the Siberian sturgeon was to spawn every two years. The spawning season for the Siberian sturgeon extended from April to May and optimal temperatures were 15ºC for females and 12ºC for males, but spawning could take place over the range 11-20ºC [176]. It would appear that sturgeon spawn in captivity, but for management reasons ovulation is induced and males and females are maintained apart to ensure tank spawning does not take place. The eggs were demersal and adhesive with a diameter of 3-3.9 mm. Spawning has been induced using single injections of carp pituitary extract (5 mg kg^{-1}), sturgeon pituitary extract (2.5 mg kg^{-1}) and GnRHa (10 µg kg^{-1}) [176], over females having an oocyte diameter greater than 2.8 mm. Male Siberian sturgeon have been stimulated with a single injection of 2 mg kg^{-1} of carp pituitary extract or 5 µg kg^{-1} of GnRHa [176].

9.10. Pleuronectiformes - Flatfish (Bothidae, Paralichlhidae, Scophthalmidae, Pleuonectidea)

The flatfishes normally undergo gametogenesis, ovulation and spermiation spontaneously in captivity, but egg spawning in females is often inhibited. Environmental manipulations are used to obtain all-year-round supply of eggs, whereas hormonal treatments are normally used to synchronize ovulations before stripping and to stimulate sperm production in males. In 2005, species from the order Pleuronectiformes had an aquaculture production of 135,512 T of which 76,884 T were from the family Bothidae, 44,666 T from Paralichthyidae, 7,124 T from Pleuonectidea, and 6,838 T from Scophthalmidae [6]. Most of the production originated from China (82,560 T), Republic of Korea (40,075 T), Spain (5,572 T) and Japan (4,591 T). The dominate species being produced were 44,666 T of the Japanese flounder (*Paralichthys olivaceus*), 6,838 T of turbot (*Psetta maxima*) and 1,445 T of Atlantic halibut (*Hippoglossus hippoglossus*).

The reproductive biology of the most relevant aquaculture species, *e.g.* Japanese flounder, Atlantic halibut and turbot, has been studied for more than 20 years, which allowed the development of efficient broodstock management and reproductive control technologies and further establishment of a successful aquaculture industry. Environmental or hormonal manipulations are normally not necessary for egg production, but they have been developed and used to improve reproductive performance and alleviate some of the encountered reproductive problems. The major reproductive disorder found in females is the inhibition of spawning, after completion of oocyte maturation and ovulation. In males, the major reproductive problems are diminished sperm volume production and production of abnormally viscous milt, which has negative consequences for both natural and artificial fertilization of the eggs [284-285]. Small milt volumes (<0.3 ml) are difficult to collect and handle, mainly if the milt is highly viscous and also, it makes difficult to avoid urine contamination, which can have deleterious effects on sperm quality and affect further fertilization rates. For a given species, these reproductive problems are found especially relevant in captive reared broodstocks in comparison to a more normal and efficient reproductive performance of wild broodstocks. For example, spontaneous tank-spawning of captive adapted wild broodstock has been described for Japanese flounder [286], turbot [287], Atlantic halibut [288] and sole [289], but further establishment of captive reared broodstock generations (F1 and successive) have shown that in most cases hand stripping of gametes and artificial fertilization is required.

Environmental manipulations are routinely used for out-of-season spawning. Normally, 12-month simulated natural photothermal cycles adequately shifted by several months in various broodstock batches is successfully used to have an all-year-round supply of gametes. Hormonal treatments are generally used to induce and synchronize ovulation in females and to enhance spermiation and milt fluidity in males. The most effective hormonal therapy for flatfishes is the single administration of GnRHa sustained release delivery systems, which has demonstrated to be highly effective in both females and males. The GnRHa implants are especially effective in female fish exhibiting group-synchronous multiple batch ovarian development, which is the case of most flatfishes [64].

From the family Paralichthyidae, several *Paralichthys spp* are of interest for aquaculture, *e.g.* the Japanese flounder in Asia and southern flounder (*Paralichthys lethostigma*) and summer flounder (*Paralichthys dentatus*) in North America. The Japanese flounder is a consolidated aquaculture species and hatchery and grow out technologies have been accomplished [286,290]. Females of this species can show spontaneous tank-spawning with adequate natural and artificial photothermal regimes, without hormonal treatment [286]. The southern and summer flounders are developing

aquaculture species [291]; for both species, all-year-round egg production, usually after stripping, can be obtained from wild broodstock using adequate photothermal conditioning and when required, hormonal treatment with slow release GnRHa pellets (100 μg kg^{-1}) [162,292-293]. Female southern flounder have been induced to ovulate with either multiple hCG injections [294] or single treatment with GnRHa cholesterol pellets, at doses of 4, 20 and 100 ug kg^{-1} [295]; in some cases, spontaneous tank-spawning has been described [171]. In the summer flounder, treatment with cPE injections, hCG injections and GnRHa implants were all effective in inducing ovulation in females, being the cPE the most effective in terms of number of ovulated females and egg fertilization rates [163].

From the family Pleuronectidae, the Atlantic halibut (*Hippoglossus hippoglossus*) is the most important aquaculture species, although some knowledge and interest exist on other species, such as, the starry flounder (*Platichthys stellatus*) and the yellowtail flounder (*Limanda ferruginea*). The starry flounder is a Pacific species and its reproduction in captivity is mainly based on artificial fertilization after stripping, which is sometimes limited by the males, because of diminished volume of abnormally viscous milt. Treatment of males with GnRHa cholesterol pellets ([D-Ala^6, Pro^9Net]-LHRH, doses of 50, 100 or 200 μg kg^{-1}), during the spawning season, increase milt volume in a dose-dependent manner, mainly by increasing milt hydration [296-297]. The yellowtail flounder is a cold ocean flatfish with aquaculture potential for the Northwest Atlantic. It is a batch-spawner, with daily egg spawns during the summer-spring spawning period. Reproduction in captivity is based on artificial fertilization of the eggs after stripping. Treatment of females with GnRHa cholesterol pellets (224 μg kg^{-1}) or biodegradable microspheres (75 μg kg^{-1}) is effective in inducing multiple ovulations, causing increased egg production, increased egg quality, synchronization of females, advancement of spawning and shortening of inter-ovulatory periods [156,298]. Similar treatments applied on males, increased sperm production and milt volume, as well as sperm motility and seminal plasma pH, while having no negative effects on sperm fertilizing ability [156,285]. The Atlantic halibut is a consolidated aquaculture species, important for North Atlantic countries. Reproduction in captivity is mainly based on artificial fertilization after stripping. Major reproductive dysfunctions are poor egg quality and unpredictable timing of ovulation in females [299] and diminished sperm production and high viscosity of the milt in males, mainly towards the end of the spawning season [300]. Another problem is the desynchronization of the broodstock towards the end of the reproductive season, when females still produce ovulations of good quality eggs but spermiation of males is drastically reduced, which make difficult the continuation of artificial fertilization trials. Environmental manipulations are used to obtain ovulation and spermiation throughout the year, by having broodstock batches under simulated 12-month photothermal regimes shifted in time several months; although successful, environmental manipulated broodstock may exhibit diminished egg and sperm quality as compared to natural broodstock [301]. Hormonal treatment of males with GnRHa implants, at doses of 5, 25, 30 and 50 ug kg^{-1}, advance the initiation of spermiation in 4 weeks and increase milt fluidity and sperm motility [284,302].

From the family Scophthalmidae, turbot (*Psetta maxima*) is the most important aquaculture species. It is naturally distributed in the Mediterranean and Atlantic coast of Europe and spawning occurs in spring-summer. Females are multiple batch spawners, each female producing up to 10 spawns per season. Simulated photothermal regimes are successfully used for all-year-round production of eggs [287,303]. Egg production in captivity is based on stripping and artificial fertilization. Egg stripping should be performed within 10 h after ovulation (at 12-13ºC) to avoid over-ripping [304]. In

captivity, turbot broodstock exhibit the common reproductive problems of other flatfishes, such as, inhibition of spawning, desynchronization of ovulation and spermiation, lack of ovulation in some female breeders and unpredictable timing of ovulation. Hormonal treatment with GnRHa pellets (25 μg kg^{-1}) is effective in inducing ovulation of 100% of the females, compared to around 50% ovulations in control broodstock and reduce by half the duration of the spawning period [164].

The family Soleidae includes several species of soles with potential aquaculture interest, mainly the common sole (*Solea solea*) and the Senegalese sole (*Solea senegalensis*). Their reproductive biology and culture techniques have been studied for more than 20 years, but their aquaculture industry has not been yet consolidated, mainly due to reproductive and pathological problems [305-307]. They are distributed in the Atlantic and Mediterranean coast of Europe, with the common sole having a more northern distribution than the Senegalese sole. The natural spawning period occurs during spring-summer, but depends on the latitude and water temperature [307]. Both species reproduce spontaneously in captivity and in contrast to other flatfishes, egg spawning is obtained spontaneously in the tank, without egg stripping [56]. Spawning is highly dependent on water temperatures. The optimal temperature range for spawning of common sole is 8-12ºC [289], whereas the Senegalese sole spawn at temperatures of 15-20ºC [306,308]. Environmental manipulations (photoperiod and temperature) are used for all-year-round egg production [289,307]. Hormonal treatments have been used to induce spawning in females of both species, mainly with the purpose of advancing and synchronizing the spawning time. Spawning of common sole have been successfully induced with GnRHa injections, at a dose of 10 μg kg^{-1} [309] and with hCG injections, at doses of 250, 500 and 1,000 IU kg^{-1} [310]. All studies on the natural and environment/hormone manipulated reproduction of sole in captivity, have shown that this is quiet easily accomplished, with high productions of good quality eggs obtained through spontaneous tank spawning [306-307]. Nevertheless, almost all information is obtained from wild broodstock adapted to captivity and as mentioned before for other flatfishes, captive reared generations (F1 and successive) may display reproductive disorders that were not detected in wild broodstock. In fact, captive sole broodstock present important reproductive disorders, mainly the absence of spawning in females and diminished sperm production in the males, which has limited to date the development of a consolidated aquaculture industry for these species.

10. CONCLUSIONS

As mentioned in the introduction and made more evident from the previous sections, fish exhibit a great variety of reproductive strategies, which must be recognized, studied and taken into account when a species in brought into captivity to function as a broodstock for aquaculture production. Characteristics such hermaphroditism or gonochorism, age-at-puberty, fecundity, internal or external fertilization, egg size and buoyancy, oviparity and ovo-viviparity, as well as parental care have important implications for finfish culture and broodstock management. Once the previous information is identified for each species of interest, the first prerequisite for the development of a sustainable aquaculture industry is the ability of the fish to undergo gametogenesis, maturation and spawning under captive conditions. It is very common for wild fish to fail to reproduce reliably when reared in captivity, but in some species this “dysfunction” is reduced or abolished with subsequent generations, as fish are inadvertently selected for the conditions prevailing in captive environment. However, other fishes never become fully “domesticated” and there is a need for

environmental or pharmacological interventions in order to control reproductive processes and induce gamete maturation and spawning. This chapter gave a general description of the fish reproductive system, its endocrine control and the reproductive dysfunctions exhibited by captive-reared fishes, and describe how this knowledge was used to develop treatments for the control of fish reproduction in aquaculture. The information presented is not exhaustive, and the reader was directed to important reviews available, but it was meant to provide the reader with the necessary background to evaluate the reproductive biology of a species of interest and to experiment with the development of reproductive control protocols.

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