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Review

Sex Control in Fish: Approaches, Challenges and Opportunities for Aquaculture

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Abstract: At present, aquaculture is the fastest growing sector of animal food production and holds great potential as a sustainable solution for world food security. The ability to control sex is one of the most important factors for the commercialisation and efficient propagation of fish species, due to influences on reproduction, growth and product quality. Accordingly, there is a large body of research that targets sexual development in commercially important species in an attempt to understand and control fish sex and reproductive function. In this review, we provide an introduction to sex determination and differentiation in fish, including the genetic, epigenetic and environmental factors that can influence fish sex ratios. We also summarise the major approaches used to control sex in fish and discuss their application in commercially important species. Specifically, we discuss the use of exogenous steroid hormones, chromosome ploidy, environmental manipulations, sex-linked genetic markers, selection for altered sex ratios, and transgenics and comment on the challenges associated with controlling sex in a commercial environment.

Keywords: sex ratio; reproductive control; triploidy; hormonal manipulation; epigenetics; environment; QTL

1. Introduction

Sex control is one of the most important and highly targeted areas of aquaculture research due to influences on husbandry management, productivity and economics. Without the ability to regulate sexual differentiation, maturation, and reproduction, farmers have little control over breeding processes, both in the hatchery and throughout grow-out. Arguably, in aquaculture species that have become global commodities, control over sex and reproduction has been the primary facilitator for large-scale industrial production. In species that are yet to reach industrial scale production, elucidation of sex differentiation and improved reliability of reproduction remains a key area of applied research.

The Need for Sex Control

Several broad goals in aquaculture can be reached through a better understanding of sex control. These include: (i) prevention of precocious maturation and uncontrolled reproduction (e.g., in tilapia); (ii) the desire to farm monosex populations due to differences in growth rate and economic value of the sexes (e.g., tilapia, shrimp); (iii) reducing the impact of phenotypic sex on product quality (e.g., Atlantic salmon, oysters); (iv) increasing stability of mating systems (e.g., sex change in groupers) and (v)) environmental and/or intellectual property protection (e.g., non-indigenous species, or genetically improved strains). The relative importance of each of these goals depends upon the reproductive biology and culture system of the species concerned.

Precocious maturation occurs in several farmed species including Nile tilapia (*Oreochromis niloticus*) [1], freshwater crayfish (*Cherax destructor*) [2] and Atlantic salmon (*Salmo salar*), which have a tendency to sexually mature and reproduce before attaining a body size that is suitable for harvest. This precocious maturation leads to slow growth as energy is diverted into reproduction, creates large variance in product size at harvest and results in overpopulation of ponds and, therefore, an inability to control animal densities and feeding rates. Furthermore, deterioration in flesh quality is often observed in female Atlantic salmon as they reach sexual maturity through the diversion of energy (e.g., lipids) towards reproductive processes resulting in differences in economic value between males and females [3]. The desire to farm monosex populations may also be provoked by sex-specific growth rates. Male Nile tilapia, for example, grow faster and have lower feed conversion rates than females [4], while female Kuruma prawns (*Penaeus japonicus*) are generally larger than males at the time of harvest [5]. As a result, farmers have adopted both manual (e.g., hand sexing and selective removal) and/or various technological (e.g., exogenous hormone treatment, chromosome ploidy manipulation, molecular tools, or hybridisation) approaches to produce monosex populations for culture (Table 1).

Table 1. Common approaches used to manipulate sex in aquaculture fishes.

Approach	Technique	Purpose	Representative example species
Hormonal manipulation	Administration of exogenous hormones (e.g., 17β-estradiol, 11-α-methyltestosterone)	Monosex	Atlantic cod, *Gadus morhua* [6] Nile tilapia (*Oreochromis niloticus)* [7]
	Administration of aromatase inhibitor (e.g., Fadrozole)	Monosex	Nile tilapia (*Oreochromis niloticus*) [8] Honeycomb grouper (*Epinephelus merra*) [9]
Hybridisation	Cross breeding	Monosex	Tilapia (*O. aurea x O. niloticus*) [10] Bass (*Morone saxatilis x M. mississippiensis*) [11]
Chromosome Ploidy	Gynogenetics	Monosex	Rainbow trout (*Oncorhynchus mykiss*) [12]
	Triploidy	Sterility	Atlantic salmon (*Salmo salar*) [13,14]
Envionmental manipulation	Manipulation of social factors	Production of male broodstock	Orange-spotted grouper (*Epinephelus coioides*) [15]
	Temperature treatment during gonadal differentiation	Monosex populations	European Seabass (*Dicentrarchus labrax)* [16–18]
Selection	Marker assisted selection (MAS)	Monosex populations	Nile tilapia (*Oreochromis niloticus*) [19,20] Turbot (*Scophthalmus maximus*) [21]

An ability to control sex and breeding is also important for hatcheries in order to produce seedstock, particularly if the purpose is reliable production of specific family combinations for selective breeding. Many fishes such as Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) can be reliably dry stripped to obtain eggs and sperm, which are subsequently mixed in buckets to produce fertilized eggs. This ability to strip spawn fish allows for easy creation of large numbers of either half- or full-sib families, depending on the mating design. However, in many other aquaculture species, strip spawning is inadequate for industrial scale production, and accordingly, natural reproduction is relied upon. Barramundi (*Lates calcarifer*) (also known as Asian seabass), for example, is a mass-spawning, sequential protandrous (male to female sex-changing) hermaphroditic species farmed throughout South-East Asia and Australia. In this species, female broodstock are extremely valuable due both to the maintenance and extensive holding time until a fish changes sex (approximately 4 or more years of age), as well as the high fecundity of female barramundi (2–32 million eggs depending on size; [22]). Although there are published accounts of dry-stripping in this species [23], industry has not adopted this approach due to unpredictable timing of sperm hydration and final maturation of eggs, as well as the risk of egg rupture and or damage to blood vessels in the ovary leading to septicemia in females used for repeated spawning [24]. As a result, hormonally induced natural mating is practiced in this species. In other species, such as sex-changing marine groupers (*Epinephelus* spp.), optimal, stable sex ratios are difficult to maintain in mating tanks due to the dependence of sexual differentiation on an individual's position within social hierarchies. For example, a fish that was introduced to a mating tank as a male may sex revert into a female, depending on its relative dominance in the tanks existing social hierarchy [25,26]. In the case of grouper, stability of males in mating groups is achieved by implants containing methyl-testosterone [27].

Given the importance of sex control to aquaculture production, the intent of this review is to introduce the major approaches currently adopted by farmers in the endeavor to have control over sexual differentiation, maturation and breeding in commercially farmed fish, as well as to highlight some of the challenges in controlling sex within a commercial aquaculture environment. Several excellent reviews on sex determination and differentiation in fish have previously been published (see [28–34] for further reading), we begin with a brief introduction.

2. Sex Determination and Differentiation in Fish

Sex determination and differentiation in fish is an evolutionarily diverse and highly plastic developmental process [33]. Such diversity leads to great challenges when trying to develop a general understanding of sex in fish. At the individual or species level, however, heightened plasticity of sexual phenotypes can lead to increased opportunity for sex control, which is particularly important for farmed fish. In many species, the sex of an individual is subject to modification by external factors, such as environmental temperature, which can interact with hormonal, genetic and/or epigenetic regulatory pathways to alter fish sexual development [35].

The term sex determination can be used to describe the genetic or environmental cue(s) that ultimately govern the sex of an individual [30]. For example, in most mammals, inheritance of the Y chromosome determines that an individual will develop as male [36]. In many reptiles, however, temperatures experienced during embryonic development, rather than genetic factors, provide the sex-determining cue. Sexual

differentiation, on the other hand, can refer to the *physical* realisation of sex determination cues, and largely pertains to the development of the testicular or ovarian tissues (e.g., phenotypic sex) that follows on from a sex-determining cue [30]. In some cases, processes of sex determination and gonadal differentiation may partially overlap and, as a result, the terms are often used interchangeably [28,34]. This is especially evident in teleost fish, in which the molecular mechanisms of sexual development are evolutionarily plastic, and require complex regulatory pathways that are governed by genetic factors (genetic sex determination; GSD), environmental cues (environmental sex determination; ESD), or on an interaction between the two (genotype x environment interactions; GxE) [37].

2.1. Genetic Factors Involved in Fish Sex Determination and Differentiation

Genetic sex determination can be grouped into two broad categories: Chromosomal sex determination (CSD), where sex is governed by the inheritance of sex-related genes located on specific chromosomes (*i.e.*, the sex chromosomes), or polygenic sex determination (PSD) in which sex determining genes are distributed throughout the entire chromosome complement, although major genetic effects are found in most cases. In fish, CSD can include both male (XX/XY) and female (ZZ/ZW) heterogamety, similar to that seen in mammals and birds. Some fish species, however, have undergone a loss of the derived chromosome (termed X0 or Z0) as in the Chilean galaxiid (*Galaxias platei*) [38], while others demonstrate translocations or fusions of sex chromosomes with autosomes (X1X1X2X2/X1X2Y) as in the wolf fish (*Hoplias malabaricus*) [39], or even exhibit multiple, fully derived chromosomes (WXZ) as in the swordtail platyfish (*Xiphophotus maculates*) [30]. In the majority of fish species, however, sex chromosomes are weakly differentiated, with only 7% of species showing sex-associated chromosome heteromorphisms [28,40]. Whilst this lack of chromosomal differentiation is suggestive of a high occurrence of PSD in fish, there are very few examples in which this has been experimentally demonstrated. These include the southern platyfish (*Xiphophorus maculatus*) [41], domesticated zebrafish [42] and likely the European seabass [43]. The lack of heteromorphism between sex chromosomes of fish may instead be explained by the inability of traditional cytogenetic techniques to identify small scale differences, such as the inversions and deletions in the Y chromosome of threespine sticklebacks (*Gasterosteus aculeatus*) [44], or the single SNP variation between X and Y chromosomes in the tiger puffer fish (*Takifugu rubripes*) [45]. Furthermore, whilst domesticated zebrafish are thought to exhibit PSD, sex chromosomes have been identified in their wild counterparts, indicating a loss, which has occurred in less than 100 generations [46]. This illustrates the rapid evolutionary turnover of sex determination systems in fish, and is an important consideration for the domestication of farmed populations.

The sex determining genes themselves may be considered as either upstream "master" switches, or downstream differentiators, depending on their relative roles in sex determination and/or differentiation. Whilst it appears that in mammals, the master sex determining gene, sex determining region-Y (*SRY*), appears relatively ubiquitous, master sex-determining genes in fish show huge variety [34]. Six master sex-determining genes have been isolated in fish thus far, including PG17: DM-domain gene on the Y chromosome (*dmY*), the major testis-determining factor in the Japanese medaka (*Oryzias latipes*) (XX-XY) [47,48] anti-Müllerian hormone (*amhY*) and anti-Müllerian hormone receptor, type II (*amhr2*) in the Patagonian pejerrey (*Odentesthes hatchery*) [49,50] and tiger puffer fish [45], respectively, gonadal somatic cell derived factor (*gsdf*) in the Luzon ricefish (*Oryzias luzonensis*) [51], sexually

dimorphic on the Y-chromosome gene (*sdY*) in the rainbow trout [52] and finally SRY-related HMG-Box gene 3 (*sox3*) in the medaka (*Oryzias dancena*) [53]. Many of these master sex determining genes are thought to have been "up-recruited" from the network of downstream genes involved more specifically in gonadal differentiation [35]. As a general model for non-mammalian vertebrates, male sex differentiation can be achieved through up-regulation of a highly conserved transcription factor, doublesex and mab-3 related transcription factor 1 (*dmrt1*), which acts in combination with transcription factor *sox-9* (*sox9*) to promote testis formation [54,55]. Alternatively, female sex differentiation is stimulated by cytochrome P450 aromatase (*cyp19a*) through a positive feedback loop involving the female-associated transcription factor known as forkhead box protein L2 (*foxl2*) [31,56]. *Cyp19a* encodes for gonadal aromatase, which catalyses the conversion of androgens into estrogens and is thought to play a pivotal role in sex differentiation and sex change in fish [31]. Other genes, such as r-spondin 1 **(***rspo*) and wnt-signalling protein (*wnt*) in the ovary, as well as *amh* and *gsdf* in the testis, are thought to play roles in β-catenin and TGF-β signaling pathways, respectively, to promote sexual differentiation and subsequent gonadal development. Precisely how these genes interact to coordinate gonadal development, particularly in the ovaries, remains a major question of sex differentiation and is likely a reflection of the variability in their relative roles between species [34,57,58].

2.2. Epigenetic Mechanisms Involved in Fish Sex Determination and Differentiation

Recently it has been proposed that epigenetic mechanisms play an important role in sex determination and differentiation in fish and other vertebrates [59]. Epigenetics is a rapidly expanding field that investigates changes in gene function that cannot be explained by changes in the DNA sequence [60]. These changes largely act to enable or inhibit the activity of transcriptional machinery on the DNA to regulate patterns of gene expression and can be influenced by environmental stimuli, such as temperature [61]. It is becoming increasingly obvious that, in many fish, differences between the sexes cannot be fully explained by differences in the genes alone, particularly in cases where sex ratio shifts occur in response to temperature, but also in serial and sequential hermaphrodites. Epigenetic modifications may be a key mechanism allowing for interactions between the environment and sex determination and differentiation processes in fish [16,59,62] (see also section 3.3 Environmental Manipulations and Opportunities for Sex Control).

2.3. Environmental Factors Involved in Fish Sex Determination

In contrast to GSD, true environmental sex determination (ESD) does not rely on sexual dimorphism at the genomic level [42]. This is because the major determinant of sex is not genetic, but environmental. The best-known environmental determinant of sex is temperature and has been well studied in reptiles that exhibit temperature dependant sex determination (TSD). In fish, the first definitive evidence for the effect of environmental temperature on sex differentiation was reported in the Atlantic silverside (*Menidia menidia*), a species which shows TSD characteristics both in the wild and in captivity [63]. TSD is also thought to exist in the high-potential South American aquaculture species, the Patagonian pejerrey, where high temperature treatments can be used to achieve all male populations [64]. In most fish, however, even the application of extreme temperature treatment rarely produces monosex populations. What is more commonly seen is an incomplete shift in the sex ratio towards either the male or female phenotype [65]. For example in the European seabass (*Dicentrarchus labrax)* temperature treatments

of 20 °C and 15 °C can be applied to achieve 73% male and 77% female populations, respectively [66]. In such cases, genotype is thought to be inhibiting complete control of the sex differentiation pathway by external factors, indicating that both genomic and environmental factors (GxE) contribute to sex determination, likely through epigenetic modifications to the DNA. The fine line between TSD and GSD + temperature effects (GSD+TE) in fish necessitates careful consideration of the ecological relevancy of temperature manipulations when attempting to classify fish sex determination systems [65]. For farmed fish, however, temperature treatments that are considered to be ecologically irrelevant can still provide important information towards the development of methods for sex control [32].

2.4. Sex Reversal and the Labile Period

Sensitivity to environmental factors in fish allows for sexual reversal, a phenomenon whereby phenotypic sex no longer corresponds to genotypic sex following experimental manipulation. For example, a temperature treated fish with ZW chromosomes (genotypically female) may exhibit testis formation as well as other male-specific secondary sexual characteristics (phenotypically male) [62]. The type and timing of treatments that are able to induce sexual reversal vary greatly between species. For example, treatments influencing sex determination and differentiation in fish include temperature, pH, density, exogenous hormone treatment, social factors, or a combination of these. Furthermore, there is a discrete time period during development, often referred to as the labile period (also sensitive window or period), during which the gonad undergoes differentiation towards the male or female state [32]. Often temperature, hormone or other treatments become ineffective following this period of differentiation, once the gonads have become established and sex is stabilised [65,67]. In the case of naturally sex-changing fish the gonads can remain responsive to either endogenous or exogenous stimuli well into adulthood. For example, social cues transmitted along the brain-pituitary-gonadal axis are thought to stimulate adult sex change in many species of reef fish [68,69]. Information on the specific cues able to influence sexual development, as well as knowledge of the timing of gonadal development and the labile period, allows for the most accurately timed and therefore effective treatments in a given species.

As the list of master sex determining genes isolated from different fish species grows, and we become more aware of the complexity of the genes in the downstream network and their interaction with the environment, it is becoming increasingly more apparent that in order to develop appropriate intervention and control strategies for sex in farmed fish, species-specific studies will be necessary. By targeting the specific developmental time periods, or environmental cues to which fish are most sensitive, such intervention can reduce the production liability of uncontrolled sex processes.

3. Approaches Used to Manipulate Sex in Farmed Fish

Given the complexity and variability in sex-determination and differentiation mechanisms in fish, there is no single approach that has proven effective in the manipulation of sex in all species. Rather sex control has relied on several major approaches including the addition of exogenous hormones, chromosomal ploidy manipulation, hybridisation (not discussed further, see [70] for a recent review), varying environmental and social parameters, and selection for sex ratios using a molecular and/or quantitative genetic approach (summarised in Figure 1).

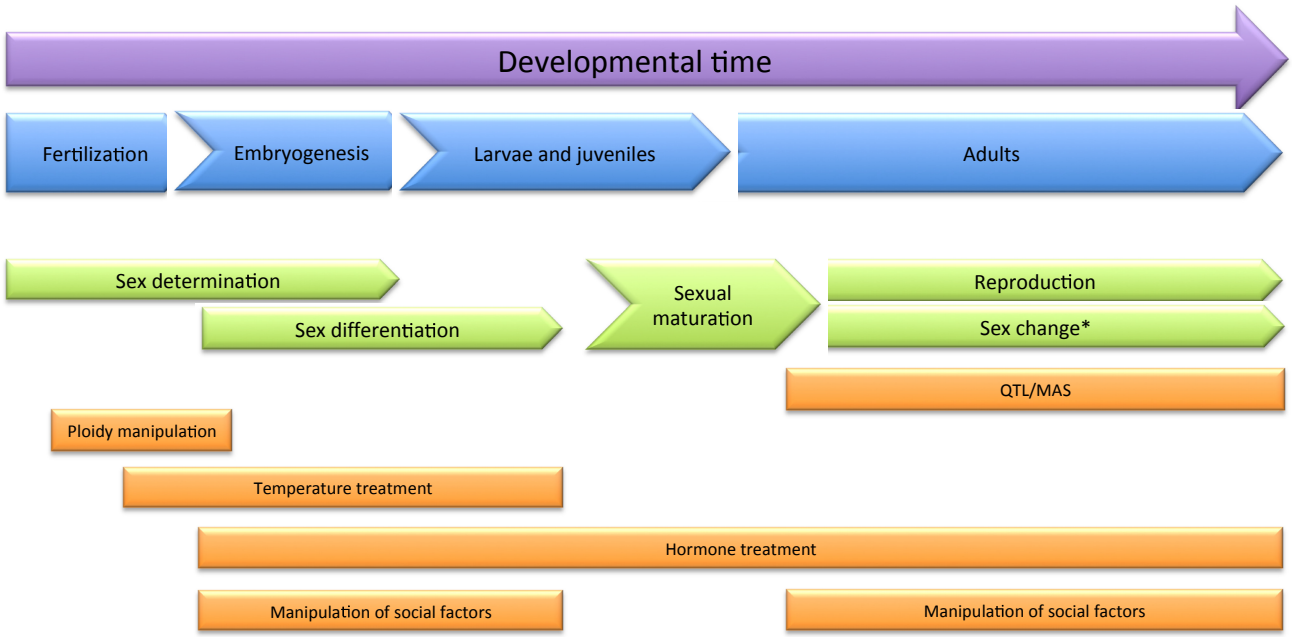

Figure 1. Generalised model of targeted developmental time periods for applying strategies to manipulate sex in farmed fish. Screening of quantitative trait loci (QTL) and marker assisted selection (MAS) is used to identify broodstock that will produce the desired sex ratio in offspring; chromosome (ploidy) manipulation to produce triploids takes place usually within 30 min of fertilisation; temperature treatments are generally best applied prior to the onset or during sex differentiation; hormonal treatments are most often applied during the period of sex differentiation, but, in some species, may remain effective throughout juvenile and adult stages; manipulation of social hierarchies are often undertaken during adult sex change (* only occurs in some fish), but can also be effective during the period of sexual differentiation of sex changing fish, or gonochoristic species where stocking density is important.

3.1. The Use of Exogenous Hormones and Other Chemicals to Control Sex

Of the external factors known to control fish sexual development, administration of exogenous sex hormones is the most frequently utilized approach due to ease of application at a commercial scale and consistency in producing monosex populations. Sex steroids (*i.e.*, those steroid hormones that are known to interact with androgen and estrogen receptors) are of critical importance to the natural process of phenotypic sex differentiation, thus providing the basis for the administration of exogenous sex steroids to alter sex ratios in farmed fish [71]. The first successful effort to artificially induce sex reversal was achieved in the medaka, through the administration of estrogens and androgens to sexually undifferentiated fish, and resulted in both functional females and males, respectively [72,73]. Following these early experiments, similar treatments have been applied to a variety of fish species demonstrating that, using sex steroid therapy, it is possible to alter the normal course of sex differentiation in fish towards the desired gonadal phenotype [74]. For instance, the addition of exogenous androgens, such as 17-α-methyltestosterone, has proven effective in the masculinization of genetically female fish in approximately 35 species [74,75]. Furthermore, hormonal treatments are not only effective in species which do not appear to possess GSD (e.g., the honeycomb grouper (*Epinephelus merra*) [74,76], red-spotted grouper (*E. akaara*) [77], barramundi

(*Lates calcarifer)* [78], but has also proven an effective treatment in species with clear chromosomal sex-determination systems (e.g., Atlantic salmon [79], Nile tilapia [7], rainbow trout [80] and Atlantic cod (*Gadus morhua*) [6]). The effect of introducing exogenous hormones prior to and during the course of sex differentiation in fish is considered successful if their addition can override normal (genetic or environmental) sex determination pathways to achieve the desired phenotypic sex.

3.1.1. Principles of Exogenous Hormones and Other Chemicals Application for Sex Control

Control of sex differentiation through the administration of hormones is generally achieved through the exposure of sexually undifferentiated fish to an appropriate dosage of exogenous sex steroids. Yamamoto [71] highlighted that for effective sex reversal sex steroids should be given prior to any sign of gonadal differentiation, and at a dosage relevant to both the species treated and the nature and/or potency of the steroid. Yamamoto [71] further recommended that hormone administration be maintained until after the time when normal sex differentiation occurs. In general, fish are much more sensitive to the effects of steroid treatment during the labile period, when the gonads are undifferentiated [81,82]. The timing of this period is variable both within and between species. For example, the labile period for estrogen treatment in the masu salmon (*Oncorhynchus masou*) is from 5 to 22 days post-hatch (dph) [83], while in coho salmon (*Oncorhynchus kisutch)* it is between 8 days pre-hatch and 13 dph [84]. Furthermore, the differentiation period of fish destined to be female often precedes that of males (because the onset of ovarian development happens earlier than testicular differentiation), such as eight days prior to hatching and 6 dph in coho salmon [84], 20 dph and 42 dph in the African catfish (*Clarias gariepinus*) [85], and 19 and 90 dph in channel catfish, (*Ictalurus punctatus*) [86] for ovarian and testicular development, respectively. Whilst hormonal treatments performed during the labile period require the minimum steroid dosage and treatment duration to produce the desired phenotypic gender [87], this does not necessarily imply that fish cannot be successfully sex-reversed by hormonal treatment outside this period [83,88]. In the case of European seabass [89] and the chum salmon (*O. keta*), the gonad is responsive to exogenous steroids outside the period of gonadal differentiation, and well beyond the labile period [83]. Nevertheless, such treatments require higher doses of hormones and longer treatment times than those that are administered during the labile period in order to successfully reverse gonadal sex.

In addition to the use of sex steroids to feminize or masculinise fish, other chemically induced sex reversal strategies are available. Among these, the use of aromatase inhibitors, such as Fadrozole, are commonly applied to induce sex change in fish. Aromatase inhibitors work by either irreversibility deactivating the aromatase enzyme (which converts androgens to estrogens), or through competitive exclusion of aromatase to receptors in estrogen producing cells. Administration of Fadrozole has proven successful in inducing complete sex reversal of genetic females into phenotypic males in Nile tilapia [8,90], olive flounder (*Paralichthys olivaceus*) [91] and honeycomb grouper [9]. In adult female honeycomb grouper, Fadrozole implanted at 10 $mg \cdot kg^{-1}$ body weight resulted in reversion of the ovaries into testes, which produced sperm capable of fertilization after two and a half months treatment [9].

Administration of aromatase inhibitors or steroid hormones to control sex in fish can occur through several routes, including direct injection of the agent into the muscle or body cavity of the fish, or more commonly, direct immersion into culture water containing the agent, or as a dietary supplement [92]. The advantages and limitations of these methods have been discussed previously by Pandian and Sheela [74]

and Beardmore *et al.* [75]. Due to simplicity of administration and applicability at a commercial scale, adding exogenous hormones via dietary supplementation is one of the most common techniques for sex reversal in aquaculture. Here, the hormone is dissolved into an evaporative solvent and added to the feed, which fish consume as part of their normal diet. The addition of hormones and chemicals to the feed has proven an effective strategy to produce 100% mono-sex populations [82,93,94], however, there are some limiting factors to this approach. These include degradation of hormone during storage or as part of the fishes normal digestive progress, variability in dosage among individuals due to non-uniformity and concentration of the hormone in the feed and behavioural hierarchies among fish that influence feeding rate and lead to differences in how much of the hormone in the diet fish may actually ingest. Whilst administration of sex-changing agents in the feed is routinely used for many species, for those species in which the gonadal labile period occurs before first feed (such as during embryogenesis, or in yolk sac larvae like most of the egg-laying salmonids [30]) immersion in water containing sex steroids offers an effective alternative. Immersion of coho salmon larvae with 400 μg/L of estradiol-17P for a period of 2 h results in clutches comprising 97% females [95]. For species in which the labile period is prolonged from embryogenesis to post-hatch, such as rainbow trout, multiple doses of steroids have been demonstrated more effective than a single dose [84,96].

3.1.2. Challenges to the Use of Hormones and Other Chemicals in Controlling Sex

Despite successful application in numerous aquaculture species, sex reversal of fish by hormonal administration manipulation should be undertaken with caution to prevent any adverse effects in the fish produced, to the farmers themselves and consumers, or to the environment. For example, hormonal overdoses or unnecessary long duration of treatments may induce deformities, or even skew sex ratios toward the non-target sex [75,93]. Within hatcheries, the use of hormones requires careful handling to avoid adverse effects on human health and hormone-laden water needs to be disposed of properly to limit environmental impacts. There are also consumer health concerns, particularly in cases where hormones are directly applied to fish destined for harvest (*i.e.*, not broodfish). In Europe, the application of hormones to commercial grow-out food fish is prohibited (with Directive 96/22/EC and Directive 2003/74/EC [97,98]), although the use of hormones to sex reverse broodfish is legal. To circumvent some of the legal, environmental and consumer issues associated with the use of sex steroids in fish, new chemicals to induce sex inversion that are regarded as safer (such as trenbolone acetate, 17α-methyldihydrotestosreone or neuroendocrine controlling substances) are being investigated [99].

3.2. Chromosome Ploidy Manipulation and Sex Control

Manipulation of chromosome-set ploidy levels (also referred to as whole genome manipulation) has been one of the most researched approaches to control sex in aquaculture species and, in several species, is routinely applied to commercial production (reviewed in [3,100–103]). Chromosome ploidy manipulation relies on the application of physical or chemical "shocks" to interfere with the normal developmental processes of gametogenesis and early post-fertilization. Depending on when these external shocks are applied, individuals that are haploid (n), triploid (3n), or tetraploid (4n) can be produced, as compared to the normal diploid (2n) somatic cell chromosome set number. Whilst there has been a body of research on producing mono-sex individuals with only a maternally (gynogens) or paternally (androgens) derived

set of chromosomes, these techniques have had little direct impact on the production of commercial aquaculture stocks. Rather, most efforts have been focused towards the production of triploids and tetraploids. Triploids are desired in aquaculture due to an inability of homologous chromosomes to pair equally in gametogenesis and the organism effectively becomes sterile. Other benefits often associated with this gonadal sterility include delays in secondary sexual maturation characteristics, along with improved flesh quality and post-pubertal body growth [3]. In contrast, tetraploids are not sterile and can produce viable 2n gametes. Tetraploids are produced for sole use as broodstock to cross with normal diploids to produce 100% interploid triploids, as is common in tilapia (*Oreochromis* spp.) [104].

3.2.1. Principles of Triploidy Induction in Fish

When fish oocytes are released from the ovary they are arrested at the metaphase stage of meiosis II. Here, homologous chromosomes have been replicated, but the second process of chromosomal partitioning to bring the gamete to the haploid state has not occurred. Meiosis then remains arrested until after the egg is fertilized by the entry of a spermatozoon [105]. Therefore, at the time of fertilization the oocyte still contains two pairs (2n) of homologous maternal chromosomes. Immediately post-fertilization, entry of the sperm causes osmotic and ionic changes leading to the resumption of meiosis II, whereby the oocyte packages up the second set of maternal chromosomes into a vesicle called a polar body which carries the additional set of maternal chromosomes out of the nucleus. This means, that for a few minutes, the now fertilized fish zygote contains three sets of chromosomes (3n), one paternal and two maternal. Physical (e.g., temperature, pressure) or chemical (e.g., 6-dimethylaminopurine, cytochalasin B, caffeine) extrinsic shocks applied after fertilization and at the time of recommencement of meiosis II can disrupt this process of polar body extrusion resulting in retention of the second set of maternal chromosomes. From this point the embryo develops through the normal process of mitotic division and embryogenesis with all its cells containing three sets of chromosomes (3n). Triploids can also be produced in some species through the initial production of tetraploid broodstock and then subsequent crossing with diploids (see [3,106] for examples).

3.2.2. Uses of Triploids in Aquaculture Production

The production and use of triploids in aquaculture has been extensively summarised in [3], where triploids from at least 49 commercial species have been produced. However, despite this large number of species the routine commercial use of this approach to control sex and achieve other production benefits is a lot lower, with the technique largely commercially restricted to use for various salmonids (*S. salmo*, *Oncorhyncuus rhodurus*, *O. masou*) and trout (*O. mykiss*, *S. trutta*, *Salvelinus fontinalis*), Artic charr (*Salvelinus alpinus*), grass carp (*Ctenopharyngodon idella*), loach (*Misgurnus anguillicaudatus*), ayu (*Plecoglossus altivelis*), edible oysters (*Crassostrea gigas*, *C. virginica*, *Saccostrea commercialis*) and tilapia [88].

3.2.3. Advantages of Triploidy Induction in Controlling Sex

The major advantage to farmers in the culture of triploids is their sterility. This is because during meiosis the trivalent homologous chromosomes cannot pair correctly during prophase I [107].

Associated with this chromosomal sterility is a severe impairment of pubertal gonad development and subsequent reproductive maturation. In females this retardation of ovarian development and maturation is usually greater than in the male testes due to the post-meiotic process of vitellogenesis in oocytes. Consequently, ovaries remain quiescent and generally are smaller in triploid individuals [108]. One example of where triploidy is routinely applied to induce sterility is that of grass carp (*C. idella*) in North America. Here, meiotic triploidy induction is used as a reproductive containment approach to produce fish that are sterile and can therefore be safely stocked into canals for weed control without the concern that this non-native species will establish feral populations [109]. Triploidy to prevent precocious and uncontrolled reproduction was also investigated initially in blue (*O. aureus*) and Nile tilapia (*O. niloticus*) [110–114]. However, due to technical difficulties in producing 100% triploids in the field, the use of sex-reversal through addition of hormones (see Section 3.1) to produce YY "supermales" and their subsequent use to produce mono-sex XY males has become a more reliable solution to the problem of uncontrolled reproduction in tilapia.

Triploidy may also delay the onset of puberty, which has further advantages to aquaculture production. The first is that the onset of puberty will result in reallocation of energy resources away from somatic growth and towards gonad maturation, gamete production and spawning specific-sexual behaviours. In some species, like Atlantic cod, feed conversion efficiency and intake may decrease with the onset of puberty. The result of these two processes is that specific growth rates may significantly decrease once puberty is reached and can be further reduced if spawning occurs (e.g., Atlantic cod may lose up to 25% of their somatic body weight through a single spawning season [115,116]). During immature reproductive phases, diploids and triploids often grow at the same rate, however, in many species once normal maturation age is reached triploids outgrow their diploid counterparts (but not always, see [3,101]). Triploid Atlantic salmon, rainbow trout, Nile tilapia, channel catfish, turbot (*Scophthamus maximus*), for example, all exhibit higher growth rates after the onset of normal puberty than diploids. An added advantage in those species where females are more desired and the sex ratio is skewed in this direction is that females outgrow triploid males [3]. Puberty and sexual maturation also may influence flesh composition through the depletion of lipids, proteins and pigments such as the carotenoid, astaxhanthin. For example, in Atlantic salmon, pre-pubertal fish exhibit higher lipid stores than in sexually mature fish, while the fillet is depleted for lipids, proteins and astaxhanthin post-maturation [117]. Finally, delaying and/or preventing sexual maturation may have an influence on reproductive behaviours that often lead to antagonistic and territorial encounters, although in an aquaculture context there is limited evidence for such alterations. Garner *et al.*, [118] found that triploid chinook salmon (*O. hynchus tshawytscha*) exhibited a slightly less aggressive feeding behaviour than diploids, although this did not result in any discernible differences in growth.

3.2.4. Challenges of Producing Triploids

Whilst farming of triploids is often seen as a panacea to control sex in fish, there are several challenges with commercial application of the approach, thus having limited adoption. Besides the obvious challenge of obtaining newly fertilized eggs within minutes of spawning and before extrusion of the second polar body (difficult in some mass spawning species like barramundi and snappers (*Sparus* spp.)), to ensure 100% triploidy it is essential to interfere with polar body extrusion in all oocytes. Inducing 100% triploids

as routine practice is, therefore difficult at commercial scales because often there is intra-specific family variability in the timing of polar body extrusion and/or oocyte sensitivity to intensity and duration of the environmental shock applied [119]. The timing, intensity/duration and best mode of shock is species-specific and in fishes to achieve 100% triploidy treatments have to cover the entire duration of polar body II extrusion and be of a sufficient magnitude to disrupt extrusion of the polar body. Thus protocols need to be individually optimized and precise in their application, as variation often leads to decreased percentages of triploidy induction success [120,121]. For instance, Piferrer, *et al.* [122] investigated the effects of cold shock timing and duration on induction of triploidy in turbot. A cold shock commencing at 5 min post-fertilisation (PF) and a duration of 20 min produced 92% triploid turbot, whereas cold shock starting at 6.5 min PF with 25 min duration produced only 83% triploids.

A further challenge in the commercial use of triploids for sex control is that often triploids exhibit lower survival, higher rates of deformities and reduced pre-pubertal growth than diploids, although this again is species-specific. As examples, triploid common carp (*Cyrpinus carpio*) had only a 70% survival rate and 85% of the growth as that of diploid controls [123], while all-female triploid Atlantic salmon, when reared in saltwater, had a survival rate of 40% compared to 60% of all-female diploids between stocking and harvest [13].

3.3. Environmental Manipulations and Opportunities for Sex Control

The influence of genotype by environment interactions on fish sex determination and differentiation allows for increased opportunity to alter fish sex ratios via environmental manipulations such as social factors, temperature, pH, and density. These manipulations can be used to alter processes of sexual differentiation during early development, or encourage sex change in adult hermaphrodite fish.

3.3.1. The Use of Social Factors to Influence Sex Change in Hermaphrodite Fish

Social interactions are commonly implicated in the onset of adult sex change in hermaphrodite fish [124,125]. Marine groupers are a group of premium aquaculture species, many of which are protogynous hermaphrodites (*i.e.*, they mature first as females and later sex change to males). A major challenge in establishing viable grouper hatcheries and breeding programs is a shortage of male broodstock [15]. Manipulation of social factors provides the possibility to attain males at a younger age. For example, to encourage female-to-male sex change in orange-spotted grouper (*Epinephelus coioides*), fish are initially stocked as mature females (~4 months) at low densities, and later transferred to higher stocking densities [25]. Social manipulations in *E. coioides* have not only been shown to influence sex ratios in adult fish, but also sexual differentiation in juveniles. By manipulating the number of juveniles reared in a single tank, Liu and Sadovy de Mitcheson [15] were able to encourage primary male maturation, with 39% of experimental juveniles maturing first as males, compared with <5% obtained under mariculture conditions.

3.3.2. The Use of Temperature to Manipulate Sex Ratios

The effect of temperature on sex ratios in fish has been documented in over 60 species, many of which are of commercial importance [30,37,63,65,126]. One of the best-studied examples of this occurs in the

European seabass. In this species, farmed populations exhibit strongly male-biased sex ratios whereas in wild populations, female biases appear more common [29]. Exposure to high water temperatures during the thermosensitive period (0–60 days post-fertilisation) has been shown to produce male-biased sex ratios through an epigenetic modification known as DNA-methylation. In this system, DNA methylation of the gene encoding gonadal aromatase, *cyp19a*, is linked to a down regulation in gene expression and subsequent masculinisation of genetically female fish [16]. Experimentally, treatments of 20 °C and 15 °C can be applied to achieve 73% male and 77% female populations, respectively [66]. Such knowledge of thermosensitivity during sexual development in juvenile European seabass offers farmers a practical tool to achieve more desirable sex ratios. DNA methylation has also been proposed as the molecular mechanism allowing for manipulations of sex ratio in farmed populations of half-smooth tongue sole (*Cynoglossus semilaevis*). This species exhibits a female heterogametic (ZW) chromosomal system, in which high water temperature is able to induce ZW males (termed, "pseudomales") [62]. It was recently observed that the promoter of the key testis-determining gene, *dmrt1*, was hypermethylated and downregulated in ZW females, but not in ZW pseudomales, and further, that these epigenetic modifications were heritable, as the epigenetic signatures of sex-reversed fish were evident in progeny reared at normal temperatures [62]. This new understanding of how temperature influences sex ratio and the underlying genetic mechanisms driving these changes has stimulated exciting and innovative research and will likely lead to new and/or optimised methods for sex control in cultured species with GSD with temperature effects (GSD + TE).

3.3.3. Other Environmental Factors Affecting Fish Sex Ratios

In addition to social factors and temperature, there are other factors known to affect sex ratios in fish that could be considered when culturing fish. For example, stocking density can strongly influence sexual differentiation pathways. This phenomenon is particularly prevalent in *Anguillidae* spp., a group of catadromous eels for which high stocking densities produce a greater number of males [127]. A reduction in stocking density is advantageous, however, where this is not economically viable, periodic transfer from high to low density can also result in higher proportions of females and increased growth [128]. Acidity of culture water may also influence sex ratios. In poeciliids, such as *Xiphophorus helleri* and *Poecilia melanogaster*, acidic waters resulted in 100% and 90% male populations, respectively [129]. Sensitivity to pH is also common in fish, such as cichlids like *Apistogramma caetei*, where 96% female progenies can be achieved at pH 6.5 [130]. More general farming practices must also be considered, for example separation of fish by size grade is often used to reduce competition and/or avoid cannibalism. However, this procedure can lead to skewed sex ratios because of the strong association between growth and sex in some fish [17,131,132].

3.3.4. Challenges for Environmental Control of Sex in Fish

There is great diversity in the types of environmental stimuli that influence fish sexual development and the effectiveness of these manipulations ranges from monosex population production (as in ESD or other highly sensitive species), to only marginal, or sometimes negligible changes in sex ratio (GxE or strict GSD). To add to this complexity, the sensitivity of sexual differentiation in a species to a given environmental cue is also highly specific [32]. For example, the European seabass is sensitive to temperature,

but not pH or density [132], tilapia are sensitive to temperature, but not salinity, density or confinement [32], and although not an aquaculture species, zebrafish appear sensitive to a broad range of factors including temperature, density and hypoxia [133–136]. An important future challenge for species of commercial importance will be to document species-specific characterisations of the environmental stimuli able to influence sexual differentiation processes, and further to identify the sensitive period during development during which treatments are most effective. Where this is achievable, environmental manipulations provide a particularly attractive technique for aquaculture production, as they provide a relatively consumer-friendly approach to sex control.

3.4. Selection for Altered Sex Ratios

Powerful and ever more accessible genomic approaches have greatly contributed to the rapid discovery of genes, gene functions and pathways associated with sex differentiation and maintenance of gonadal state in commercially important fish species [34]. The use of next generation sequencing and high throughput SNP genotyping technologies have allowed for high-resolution linkage mapping and quantitative trait loci (QTL) studies and their potential use in marker assisted selection (MAS) breeding schemes [137]. Although identification of sex-specific markers in fishes has traditionally been performed with low throughput random amplified polymorphic DNA (RAPD) markers (e.g., sex-specific DNA markers #1 and #2 from African catfish, *Clarias gariepinus*, *CgaY1* and *CgaY2* respectivley [138,139]), high density marker genome screening using restriction site associated DNA (RAD) markers are enabling the rapid identification of sex-associated loci and the development of sex-specific markers in species with genetically determined systems (e.g., Nile tilapia X-linked *Oni*23063 and *Oni*28137 SNP markers [140] and *Oni3161* in linkage group 20 [141]). The recent discovery that the underlying mechanism determining sex in fish may not be exclusively related to sex-specific genes (or alleles) themselves, but in fact related to epigenetic modifications, has resulted in a broadened perspective of where and how we should be looking at the DNA [59]. Nonetheless, the three complimentary fields of genome mapping [142], epigenetics [35] and selection for altered sex ratios [143] are likely to deepen our understanding on how to effectively manipulate and control sex differentiation in fishes.

3.4.1. Heritability and Potential for Selection of Altered Sex Ratios

For selective breeding to be effective, biases in sex ratio in a population must be (i) a quantitative genetic trait and (ii) under additive genetic control (heritable). As yet, there is very little information on the heritability of sex ratios in most commercial species, and for those species for which data do exist, heritability for biased sex ratios has been shown to be nearly 2-fold that of the most selected trait in aquaculture, growth (e.g., Nile tilapia: $h^2_{sex} = 0.77$ *vs.* $h^2_{weight} = 0.31$ [19,144], rainbow trout: $h^2_{sex} = 0.67$ *vs.* $h^2_{weight} = 0.37$ [145,146], European seabass: $h^2_{sex} = 0.62$ *vs.* $h^2_{weight} = 0.41$ [43,147]). These data suggest that within a population, individuals are genetically inclined to produce families with either more male, or more female offspring. Indeed, trials with Nile tilapia have shown that sex ratio can be selected [20]. Male Nile tilapia originating from a family comprised of >90% males produced progeny with equally biased sex ratios (85%–100%), regardless of whether they were mated with females from families with balanced, or male biased sex ratios [20]. However, selected offspring did not always produce similarly biased sex ratios to parents, suggesting that in some cases, the trait was not inherited [148,149]. Wessels

and Hörstgen-Schwark [144] later showed that sex ratios may be influenced not by the sex-determining genes themselves, but their thermo-sensitivity (because sex ratio in families exhibited changes dependent on whether 9 day old progeny were exposed to 36 °C for 10 days). Over three generations of divergent selection for a high male line (families with >80% male offspring) and a low male line (families with <60% male offspring), Wessels and Hörstgen-Schwark [143] observed changes in the proportion of males to 92.7% and 50.4%, respectively. The authors also showed that for sex ratios to be selected for progenies must be exposed to temperature treatments during the thermo-sensitive period, suggesting an epigenetic mechanism is also at play. Similar results to tilapia have since been found within a single generation of rainbow trout [145].

3.4.2. The Use of Quantitative Trait Loci to Improve Sex Control

When variable sex ratios within a species exist within a population, or among different families, due to both genetic and environmental effects, sex can be regarded as a quantitative threshold trait [43,150]. In such instances, QTL mapping can assist in determining the number and effect of chromosomal regions determining sex, as well as to narrow down regions of the genome where major sex determining genes are located [137]. QTLs have been identified for various phenotypic traits, including sex traits, in over 40 aquaculture species [137,142]. Most of these efforts have been directed towards finding sex, or early maturation QTLs in species such as tilapia, sea bream *Sparus aurata*, rainbow trout, turbot [21] and others [140,151–156]. In Nile tilapia, for instance, QTLs for sex-determination have been detected on linkage groups 1 and 23, with the key sex gene *amh* residing within the middle QTL of linkage group 23 [153]. In turbot the *Sma-USC30* microsatellite on LG5 has been identified and used in commercial hatcheries as a precocious sex marker and to establish expected sex ratios in progeny [157]. Ultimately, QTL mapping aims to provide useful sex-linked and temperature-dependent sex reversal markers as a tool to accelerate the genetic improvement of monosex populations through selective breeding programs [140,152,155,158,159]. Alternatives to such DNA markers include a number of transcriptomic approaches, which aim to screen for genes that are differentially expressed between testis and ovaries [35]. The use of RNA-Seq technology to identify sex-biased gene expression has greatly contributed to the identification of major coding sequences underlying sex-determination/differentiation pathways in an increasing number of aquaculture species such as the Asian sea bass [160], olive flounder [161], sharpsnout seabream (*Diplodus puntazzo*) [162] and African cichlids [163].

4. Other Methods and Future Directions

Recent developments in genetic technologies have introduced the opportunity to control sex through the direct targeting of genes involved in either sex determination and/or reproductive maturation. Two of these technologies, namely antisense RNA gene knock-down and insertion of transgenes to achieve transgenic sterilisation hold greatest potential for commercial application [164]. Despite this, there are few examples of their application to control sex at this time and this paucity of examples is likely due to an inefficiency in achieving complete and reliable outcomes [165]. Whilst this inefficacy may be due to technical issues, there also are regulatory hurdles that need to be overcome. Regardless of these issues, preliminary work on controlling sex via direct targeting of sex and reproductive genes has commenced in several species including tilapia, rainbow trout and common carp (*Cyprinus carpio*) [166–169]. Studies

with these three species have targeted key reproductive hormone genes like gonadotropin-releasing hormone (GnRH), a key hormone released from the hypothalamus stimulating the release of pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which signal the gonads to differentiate and mature. In rainbow trout, for example, Uzbekova *et al.* [166] were one of the first to employ a single-stranded antisense RNA silencing approach using DNA from the Atlantic salmon GnRH3 gene. In this experiment, *GnRH* mRNA in transgenic rainbow trout was down regulated, however, the effect on FSH and LH levels was not significant. Despite this, Uzbekova *et al.* [166] showed that maturation among treated fish showed increased variability than those in the control group. Similarly in tilapia, females with a GnRH3 construct under the control of a constitutive common carp *β-actin* promoter were half as fertile as control females [167]. Whilst these studies using transgenic anti-sense technology were not able to induce complete sterility, they highlight the potential of this approach to control sexual processes in aquaculture fish species in the future.

5. Concluding Remarks

Sexual development is a complex, often species-specific process in fish. Therefore, technological innovations that successfully control sex in one species are certainly not guaranteed to work in another. Despite this complexity, there is an armory of approaches available to farmers to enable them to influence sex in their culture species, and many of these have now achieved commercial implementation. Our knowledge of the genes involved in sexual development and the mechanisms by which environmental modifiers can induce phenotypic changes is also rapidly increasing, due to the ease with which whole-genome sequencing and transcriptomic studies can now be conducted. Incorporation of current approaches to control sex with this new genetic (and epigenetic) understanding will undoubtedly lead to further advances in sex control of fish and will be a significant catalyst for selective breeding and the culture of more productive populations of farmed fish in the near future.

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Conflicts of Interest

The authors declare no conflict of interest.

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