Chapter 6
Monosex in Aquaculture

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Abstract Monosex refers to the culture of either all-male or all-female populations, a sought after approach in aquaculture. This chapter reviews the advantages of monosex population culture and details the mechanisms to achieve it based on different modes of sex determination and sexual differentiation. A recent case study for an aquaculture biotechnology based on sexual manipulation in crustaceans serves in this chapter to identify the key elements for a successful application. This application which makes use of RNA interference with a key regulating hormone opens the pathway toward environmentally friendly applications in fish and additional aquacultured species. This chapter portrays the state of the art in sexual manipulations in aquacultured species, starting with vertebrate species, followed by the case study of the crustacean species and discussion on how the techniques used in this study are applicable for other species.

6.1 What Is Monosex?

Sexual dimorphism (or gonochorism, where two predominant forms exist with different characteristics between males and females) is a common phenomenon in many sexually reproducing animals. One of the most extreme cases of sexual dimorphism is evident in the triplewart seadevil anglerfish (*Cryptopsaras couesii*) where the mature female reaches up to 30 cm in length, while the parasitic male is as small as 1 cm, born with no digestive system and with an extraordinary sense of smell. The male anglerfish perceives the pheromones secreted by a reproductively active female as food and rushes to feed on her flesh and while doing so secretes enzymes which consume both him and part of the female, leaving only the testes intact, connected by anasthomoses to the female's body (http://www.fishbase.org/

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summary/3098). If this species had been used in aquaculture production for human consumption, production of all-female populations would be orders of magnitudes more beneficial than mixed population culture. While sexual dimorphism is not as extreme in many commercially valuable species, there is still advantage for monosex culture of either all-male or all-female populations in many species, for different reasons, depending on the species and the market.

6.2 Why Monosex?

Monosex population culture is desired for several reasons. In most species there are sexually dimorphic patterns of favorable traits that are more accentuated in either males or females. In the more established poultry industry, for example, the production of all females for eggs and the production of all males for meat are desirable. So is the case with milk and meat in cows and bulls. In general, most fish species show dimorphic growth rates. Males grow faster in some species (like tilapia and catfish), while females grow faster in other species (like grass carp, several salmonids, and cyprinids). Monosex population culture of the faster growing gender therefore increases the production rate. When culturing one sex, the culture conditions are tailored to better suit the energetic demands of the cultured gender. Also, since there is no unwanted breeding, the genetic breeding program is managed in separate compartments and is more controlled. If unplanned reproduction was to occur in grow-out ponds, it would result in crowding and higher densities than intended and also lead to energy waste due to sexual activity on the expense of growth. The end product of monosex culture will therefore be more uniform as a consequence, a crucial marketing advantage. Introducing culture of advantageous exotic species is met with resistance or is not accepted in some countries due to the ecological risk (Ventura and Sagi 2012). One such example is Mozambique tilapia (*Oreochromis mossambicus*) that is an important aquacultured species worldwide, which invaded Queensland waterways and is considered there a noxious species. The ability to introduce one gender could mitigate the risk of leakage to natural waterways. Even if leakage should occur, it will not be viable for more than one generation. Last, theft of genetically improved lines is not optional when only one gender is marketed.

6.3 How to Generate Monosex?

Today monosex culture is being practiced in many fish species as well as in several invertebrate species either by manual segregation or, primarily by sex reversal, directly to the desired sex, or the other way around, through sex reversal to the undesired sex which will produce the desired sex in its progeny. While manual segregation requires the least technology, it is tedious, wasteful, and inefficient. Direct sex reversal requires administration of substantial amount of hormones to

skew the ratio in the population toward the desired sex. Indirect intervention is most beneficial and involves hormone administration to the parents, while the progeny product is not treated.

What prior knowledge is needed to generate monosex populations?

First, the genetic background needs to be clarified: Are male heterogametic with two non-similar sex chromosomes designated XY, or are they homogametic with two similar sex chromosomes designated ZZ (where females are the heterogametic sex with ZW sex chromosomes)? What is the preferred gender? The latter factor is not only species-related but also market-related, as for some species certain markets will prefer males and other markets will prefer females (Ventura and Sagi 2012). What are the molecular mechanisms of sex determination and sexual differentiation? These mechanisms vary between species of the same genus, making it hard to trace key effectors that can be manipulated. Still, there are key elements that are somewhat conserved across species that can be targeted. What is the timing for appearance of sex traits and what is the timing for successful sex reversal? Usually, the appearance of sexual traits marks the stage at which it is too late to intervene and enable a functional sex change. For this reason, sex markers are essential to discriminate between a successful sexually reversed individual and a false-identified one (Ventura et al. 2012).

6.4 Sex Determination and Sexual Differentiation

Sex determination and sexual differentiation are defined as two overlapping processes. These processes are usually governed by a master regulator that regulates a major effector that initiates an entire cascade of genetic network that leads to the onset of the characteristic phenotypes. Sex determination is set genotypically (with the exception of temperature or other environmental cues such as social structure that govern sex determination in some species), while sexual differentiation is the onset of the phenotypic changes which follow the effect of the sex determination process. These are overlapping processes which can be manipulated using the correct timing and tools, if available. Sex determination and sexual differentiation have evolved numerous times throughout evolution. For this reason many mechanisms are in place to reach similar outcomes in sexually reproducing species. In fact, sex is quite plastic in the animal kingdom with some species that start as males and then turn into females (protandry) or vice versa (protogyny). Additionally, some species are capable of self-fertilizing and have both testes and ovaries (hermaphrodites). The aquaculture-relevant fish species that relate to each form of sex determination were extensively described (Devlin and Nagahama 2002; Pandia 2012). To simplify the discussion into monosex applications, we will focus on genetic sex determination and species with two distinct sexes (gonochoristic).

6.4.1 Sex Determination: Heterogamecy

Sex determination starts right at the beginning with the fertilization. In case of a genetic sex-determining system (as opposed to environmental regulation), the sex chromosomes contributed by the gametes define the sex. In many aquacultured species, the sex of the progeny is defined by the Y or X sex chromosome carried by the sperm cell, like in humans. This means that males are the heterogametic sex (XY), while females are the homogametic sex (XX). In many other aquacultured species, sex of the progeny is defined by the W or Z sex chromosome carried by the egg. This means that females are the heterogametic sex (ZW), while males are the homogametic sex (ZZ). In many phyla it has been shown that species of the same genus could have either XX/XY or ZZ/ZW sex determination mechanism. For that reason manipulation of sex for monosex population culture is a case-by-case issue. In very few species, the sex determination mechanism has been studied to the level where it presents opportunities to manipulate sex very early during development, with the initiation of the sex determination cascade. This includes the conserved DM domain genes (*Dmrts*), which are related to the insect gene *doublesex* (*dsx*) that are located on the sex chromosomes in a handful of species, anti-Müllerian hormone gene located on the Y chromosome (*Amhy*) in the Nile tilapia, *Oreochromis niloticus*, and sex-determining region on the Y chromosome (SRY) in mammals. These factors are evolving rapidly, making it hard to identify, and in many cases they include homologues that are not sex-linked (Sandra and Norma 2009).

6.4.2 Sexual Differentiation

Sexual differentiation is the process that leads from an undifferentiated zygote to the differences between males and females. The molecular basis for sexual differentiation is highly variable between species. As an example, sex-lethal (*Sxl*) acts upstream of the sexual differentiation pathway in one of the most studied invertebrate model species *Drosophila melanogaster*. Homologues for *Sxl* were identified in many additional dipteran insect species, in none of which does *Sxl* assume a sex-determining role (Suzuki et al. 2001). *Sxl* differs between males and females. While the male isoform is inactive, the female isoform is an active splice factor which leads to the expression of the female-specific *transformer* (*tra*, a splice factor as well), which then leads to alternatively spliced *doublesex* (*dsx*). While *tra* does not retain its sex-determining role even within the genus (Suzuki et al. 2001), *dsx* retains its sexual differentiation relevance across phyla. For example, the homologous gene in the nematode *Caenorhabditis elegans* (called *mab-3*) encodes a DM domain transcription factor that controls male development. Similarly, the vertebrate Doublesex and mab-3 related transcription factor 1 (Dmrt1) is a DM domain-containing transcription factor that governs sexual differentiation in a wide array of species (Raymond et al. 1998). Downstream from these factors (that are

considered sex-determining factors that govern sexual differentiation), the sexual differentiation cascades converge between phyla and include several conserved factors. For example, amh and the sex steroid machinery (which includes the enzymes that metabolize the steroids, the steroid secretion system, and their receptors) are conserved in vertebrates (Sandra and Norma 2009).

6.5 Hormonal Regulation of Sex Differentiation in Vertebrates

Regulation of sex determination in several species is influenced by environmental factors such as temperature, but in most species it is regulated genotypically. In species where genetic regulation controls the gonad development, a master gene starts a chain of events leading to expression of different genes at different levels and timing, causing the differentiation of the gonad (Pandia 2012). The region where the gonad develops in mammals and cartilaginous fish is called the urogenital ridge as it is a region that gives rise to both the gonads and the urinary system. Initially the gonad is undifferentiated and is "bipotential," in the sense that given the right cue, it can develop either way. The bipotential gonad has two sets of ducts named the Müllerian ducts that can develop into oviducts and Wolffian ducts that can develop into sperm ducts. At a certain stage, the gonad cells start proliferating and secrete anti-Müllerian hormone (Amh) and testosterone and express Dmrt1 and the transcription factor Sox-9 (which promotes Amh expression), leading to testis differentiation. When the signal is not strong enough, an array of other signals will later be expressed following meiosis, leading to the expression of aromatase which converts testosterone to estradiol and the outcome of such event will therefore be differentiation into an ovary (Sandra and Norma 2009; Pandia 2012). In teleost fish, the bipotential gonad does not include two sets of ducts, but one set of ducts (Wolffian) that can develop either way, depending on the presence of the genetic factors described above. Since there are no Müllerian ducts in the developing gonads of teleost fish, it is not clear what the role of Amh is. This is a generalized description of the process (depicted in Fig. 6.1) that has many species-specific variations (Pandia 2012).

The active Amh is a large glycoprotein homodimer of 140 kDa, stabilized by disulfide bridges. While encoded by an autosomal gene in most studied vertebrates, it is encoded by a gene that is located on the Y chromosome in the Nile tilapia (*Amhy*). Amh, also called Müllerian-inhibiting substance (Mis), is a glycoprotein secreted by the Sertoli cells of the fetus (Munsterberg and Lovell-Badge 1991). Amh can be considered as a safe target to use for sex reversal, as it contains species-specific sequences. Testosterone, on the other hand, is produced through steroidogenesis and is generic for many vertebrate species. The use of testosterone or its feminine counterpart estradiol or derivatives thereof is thus environmentally unsafe.

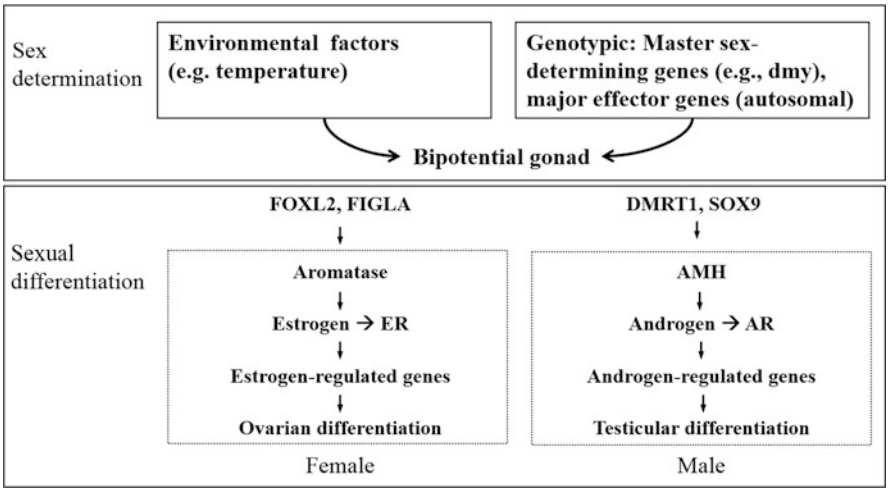

Fig. 6.1 The key steps in sex determination and sexual differentiation in vertebrates. Sex determination starts with an environmental factor or a genetic factor. In the case of a genetic factor, a master sex-determining gene is defined as the gene that resides on the sex chromosome and initiates the cascade. The major effector gene can be autosomal, and it is the key factor that leads to the translation of the genetic constituent into a sexual differentiation cascade. At the bipotential gonad, different factors are expressed between males and females, leading to sexual differentiation. In females (left), transcription factors like forkhead box protein L2 (FOXL2) and folliculogenesis-specific basic helix-loop-helix protein (FIGLA) express early during ovarian development and are important for its function. In males (right), transcription factors like DMRT1 and SOX9 are important for initiating the male-specific cascade including AMH and biosynthesis and secretion of androgens that bind to their receptors (AR) to initiate a cascade of androgen-regulated genes. In females, aromatase converts the androgens to estrogens which bind to their receptors (ER) and initiate a cascade of estrogen-regulated genes

6.5.1 Steroidogenesis

Steroidogenesis is a continuous process of converting the precursor molecule cholesterol into an array of steroid hormones by various enzymes that metabolize it. Steroidogenesis is taking place both in males and females in the gonad as well as additional tissues. Different enzymes convert cholesterol to derivatives known as progestogens followed by a set of enzymatic reactions which convert them to androgens, which is followed by yet another set of enzymes which convert androgens to estrogens. The process is species-specific and includes various androgens and estrogens in different species. Androgens regulate masculinity, while estrogens regulate femininity. It is therefore not surprising that one key enzyme in the sex differentiation process in vertebrates is the aromatase which converts the androgenic hormone testosterone to the estrogenic hormone estradiol (Bao and Garverick 1998). Aromatase is used as a sex marker, since it is expressed in much higher levels in females and can be found in peripheral tissues. Still, it is not as reliable as genetic

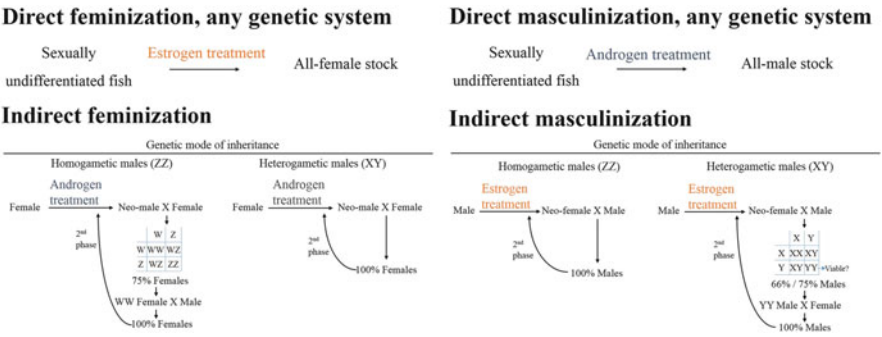

Fig. 6.2 Direct (top) and indirect (bottom) sex reversal from male to female (left) or female to male (right) using androgens and estrogens in vertebrate aquacultured species. While direct manipulation does not require prior knowledge of the genetic mode of inheritance, it affects the indirect approach

DNA markers, and the search for these is still ongoing for many aquaculture important species.

6.6 Sex Reversal Induction in Vertebrates

In order to produce monosex populations in vertebrate aquacultured species, usually synthetic estrogens or androgens are administered to induce sex reversal. This method is not environmentally friendly since these compounds end up in waterways (Megbowon and Mojekwu 2014). In several species temperature manipulation proves useful to some extent, although it does not hold for most species. Feminization can be induced directly through administration of estradiol or indirectly through administration of androgens which results in all females through either WW or XX neo-males (functional males that carry a female genetic load). The other way around, masculinization can be induced directly through administration of androgens or indirectly through administration of estrogens which result in either ZZ or YY neo-females (Budd et al. 2015). The options for direct or indirect routes of sexual manipulations, considering the genetic mode of inheritance are depicted in Fig. 6.2. For direct manipulation, prior knowledge of the genetic mode of inheritance is not required, and it is thus simpler. The downside is that this mode is more costly and less ecologically beneficial, since all individuals in the population must be treated, while in the indirect manipulation route, only the brood stock needs to be treated. Indirect feminization is simpler in XX/XY species, as it leads directly to all-female populations, due to eliminating the Y chromosome from the lineage. In ZZ/ZW systems, on the other hand, feminization is more complex as neo-males (ZW) are mated with females (ZW), and only following a second generation, one can isolate the WW females that enable all-female population. Indirect masculinization is simpler in ZZ/ZW species as neo-females are (ZZ), and crossing them with males (ZZ) results in all-male population. In XY/XX species, indirect masculinization is

more complex as neo-females are XY and crossed with XY males. Then the ratio of males in the population through such a cross will be either about 75% or 66.7%, depending on whether YY individuals are viable or not. If they are, the 75% males are expected. Then, these Y males can be isolated and crossed with females to produce all-male populations in the second generation. In order to eliminate the X chromosome from the lineage, one must keep performing indirect feminization on these YY males.

To summarize, the direct route for gaining monosex populations of aquacultured vertebrates is simpler but less eco-friendly. In rainbow trout direct feminization is used since females reach sexual maturity later than males, grow faster, and have superior flesh quality. Yet, there is an issue with incomplete sex reversal that in some cases generates hermaphrodites with ovotestes. Direct masculinization through androgen administration is practiced in Nile tilapia due to faster growth of males in this species. Grass carp is being indirectly feminized as females grow faster in this species. Instead of using feed pellet that includes androgens, the technique used to induce sex reversal in grass carp that does not eat artificial feed is implanting slow release implants with the androgens. Indirect feminization is practiced in salmon due to faster growth rates of the females.

6.7 The Case of *Macrobrachium rosenbergii*: The Commercially Most Important Freshwater Prawn

The global aquaculture trend over the past few decades shows that while capture fisheries are maintained at relatively the same level, aquaculture keeps on growing, dictating the need for better aquaculture techniques to keep up with the growing market demands. In the giant freshwater prawn *Macrobrachium rosenbergii*, this trend is much steeper, necessitating a more immediate solution for improved aquaculture techniques. *M. rosenbergii* males grow faster than females and reach higher weights at harvest. With that, they show a hierarchical population structure and are thus more spread across various sizes as compared with females that grow more uniformly. This species is thus an example for how either all-male or all-female populations are preferable compared with mixed populations, depending on the market demands (Cohen et al. 1988). It was shown that selective harvest of either all-male or all-female population gains higher yields, pointing to the economic advantages of prawn monosex aquaculture. Manual segregation is a common procedure in crustacean aquaculture to separate males and females into separate ponds. Although profitable, this is a labor-intensive, tedious process (Nair et al. 2006). A biotechnological approach has been thus developed, harnessing knowledge of the sex determination and sexual differentiation mechanisms. In crustaceans, sexual differentiation toward maleness is dictated by the circulating androgenic gland hormone, secreted by the male-specific androgenic gland (AG) (Ventura et al. 2011b). This gland governs masculinity development and maintenance. In

M. rosenbergii mating normally yields 1:1 sex ratio. Upon AG removal from males at a very early developmental stage, a full and functional sex reversal is achievable from males into neo-females (Sagi and Cohen 1990). Since *M. rosenbergii* males carry two homologous sex chromosomes, designated ZZ, the progeny obtained by crossing neo-females with males is 100% males. This process takes a long time, requires skilled surgeons, and results with very low success rates. Similarly, grafting of AG tissue or cells into females at a very early developmental stage results with neo-males that can generate all-female populations (Malecha et al. 1992). In both cases the success rates were extremely low, up until recently when genetic sex markers were identified (Ventura et al. 2011a).

The significance of the sex markers lies in the fact that for a successful intervention, the manipulation must be done in early developmental stages, prior to the emergence of sexual characteristics (Ventura et al. 2012). This means that 50% of the treated individuals are males and 50% are females. Following manipulation, for example, by AG cells administration, we expect more than half to develop as males. That means that 50% are males and the rest are neo-males. How can we tell them apart? Without sex markers we will have to grow all treated individuals until we can see if they develop into males or females, then discard unsuccessful attempts (in the case of indirect feminization, the females, as we would like to produce neo-males), mate the suspected neo-males with females (a resource demanding process that could take up to a year), grow the progeny of each and every individual cross independently until they show sexual characteristics (up to another 6 months later) and based on the ratio of females to males, decide if the progenitor is indeed a neo-male (bearing ZW sex chromosomes) or a male (ZZ). Using the recently identified sex markers, the need for the tedious validation is alleviated. All that is required is a small piece of tissue from each manipulated individual and within a day all neo-males can be separated with high degree of accuracy.

Using a cDNA library of the AG, an insulin-like encoding gene which is expressed specifically in the AG was identified, termed *Mr-IAG* (Ventura et al. 2009). Like other insulin-like peptides, mature *Mr-IAG* is hypothesized to comprise a B chain linked by two disulfide bridges to an A chain where another disulfide bridge occurs. The time when *Mr-IAG* expression initiates is prior to development of any sexual characteristics, establishing the correct timing for successful sexual manipulation (Ventura et al. 2009). Repetitive injections of dsRNA of *Mr-IAG* into juvenile male prawns resulted with RNA interference (RNAi) and knockdown of *Mr-IAG*, giving rise to a full and functional sex reversal into neo-females (Ventura et al. 2012).

6.8 Sex Reversal Induction in Other Crustaceans

Decapoda are the order of ten-legged crustaceans that comprise the aquaculture-relevant species like crabs, crayfishes, lobsters, prawns, and shrimps. Over the past decade, the IAG was identified in more than 20 decapod species, covering

representatives of all major cultured groups (Ventura et al. 2011b, 2014). Attempts to establish all-male or all-female populations in additional crustacean species are ongoing for several commercially important species. The attempts rely on genetic sex markers where available, for a handful of species, and also the IAG, or the AG itself. Additional technologies might include recombinantly produced IAG or a nucleotide vector that will express it. In some cases, the research has led to novel findings, as in the Eastern spiny lobster *Sagmariasus verreauxi*, where novel insulins were identified (Chandler et al. 2015) as well as genetic sex markers that are also the master sex-determining genes.

6.9 Concluding Remarks

In summary, IAG is considered to be the key regulator of sexual differentiation in crustaceans. The case study of the giant freshwater prawn serves as a model for sex reversal through AG manipulation or IAG gene silencing. These methodologies are environmentally friendly since the intervention is species-specific, and unlike the practice common in vertebrate species, it does not include hormonal treatment that might affect other organisms. The sexual steroids are not available in crustaceans, a fact that was driving the research to the key sexual differentiating insulin-like hormone, which has led to a safe approach for sexual manipulation that can be translated to similar approaches in vertebrates. As an example, some of the key vertebrate factors highlighted in this chapter, like amh and Dmrt1, could be utilized in a species-specific way to develop novel techniques for monosex population aquaculture biotechnologies.

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