Puberty in Teleost: Biotechnological Applications

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INTRODUCTION TO PUBERTY IN FISHES

Freshwater fish, such as Tilapia, Rohu, Catla, Carp, Mrigal, and others, play a vital role in the economies of many countries, serving as a staple food for a significant portion of the population. Due to their importance, the aquaculture sector has been rapidly expanding as a sustainable solution to address global food security concerns, particularly in animal food production. In fact, the freshwater aquaculture industry now contributes to over 95% of the overall aquaculture production [1].

The growth of the aquaculture sector relies heavily on the proper breeding and reproduction of fish. In India, fish breeding periods vary, and reproduction occurs periodically, governed by a combination of external environmental factors and internal regulatory mechanisms.

The crucial internal regulatory factor governing reproduction is the molecular mechanism known as the central reproductive axis or the Hypothalamic-Pituitary-Gonadal (HPG) axis. Within this axis, the release of Gonadotropin-releasing hormone (GnRH) from the hypothalamus triggers the production and release of pituitary glycoprotein hormones called gonadotropins, namely luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These gonadotropins then act on the gonads, promoting gametogenesis and steroidogenesis by stimulating the secretion of sex steroids, such as androgens and estrogens. Positioned at the pinnacle of this axis, GnRH neurons regulate reproduction by integrating information from social and environmental cues in conjunction with the hormonal status [2].

Puberty represents a biological transition that occurs in vertebrates, marking certain physiological changes in their bodies. During this period, individuals exhibit the first signs of sexual maturity and become capable of reproduction. The onset of puberty in vertebrates, including fishes, coincides with the activation of the HPG axis. In teleosts, puberty typically begins after gonadal sex differentiation [3], characterized by the commencement of spermatogenesis in males [4] and vitellogenic ovarian development in females [5].

In the context of aquaculture, comprehending the mechanism responsible for initiating puberty and the diverse factors influencing this process is crucial for effectively managing the onset of puberty in fish farming. The biotechnological advancements in studying the puberty in fish are playing a significant role in advancing our understanding of the underlying mechanisms. Some of the key biotechnological approaches that are being utilized are discussed below.

1. Transcriptomics, Genomics and Proteomics

High-throughput sequencing technologies like RNA sequencing (RNA-Seq) and whole-genome sequencing have enabled researchers to study the gene expression patterns and genomic changes associated with puberty in fish. By comparing the transcriptomes and genomes of fish at different developmental stages, scientists can identify genes and pathways involved in the process.

With the help of transcriptomics and genomics, internal determinants have been identified in teleosts during the pubertal onset time. Kisspeptin, encoded by *kiss1* gene, has been identified as the gatekeeper of the HPG axis by breakthrough researches conducted in 2003. It was noticed that a mutation in the *kiss1r* gene (receptor of kisspeptin) led to delayed puberty and a condition called hypogonadotropic hypogonadism in humans [6, 7]. Later on, the gene was then identified in different fish species like Rohu (*Labeo rohita*) [8], Zebrafish (*Danio rerio*) [9], European Sea Bass (*Dicentrarchus labrax*) [10], Goldfish (*Carassius auratus*) [11], Chub Mackerel (*Scomber japonicas*) [12], Catla (*Catla catla*) [13], Medaka (*Oryzias latipes*) [14] and Senegalese Sole (*Solea senegalensis*) [15], where the levels of *kiss1* were found to be increasing sharply during the pubertal onset time. Growth factor like IGF-1 (insulin-like growth factor-1) plays a role in progression of puberty in teleosts as it was found to directly stimulate the synthesis and release of the growth hormones [16, 17]. Leptin, a hormone produced by adipocytes, serves as a signal that communicates the accumulation of lipid stores and the initiation of puberty [18]. Its role in controlling the pubertal onset has been identified in several fishes like Chub Mackerel [19-23], Blunt Snout Beam (*Megalobrama amblycephala*) [24], European Sea bass [25] and Rainbow Trout (*Onchorynchus mykiss*) [26] where the transcriptomics data showed that it regulates the gonadotropin secretion and gonadal maturation. Neuropeptide Y (NPY) and Neurokinin B (NKB) have also been identified to stimulate the gonadotropin and growth hormone release in Tilapia [27] and Zebrafish [28].

In order to investigate the miRNAs that control mRNA expression during the onset of puberty in Atlantic Salmon, an integrated transcriptome analysis of salmon testis was conducted at three different developmental stages: immature stage, pre-pubertal stage and pubertal stage. During the immature stage, the analysis unveiled miRNAs that were already known to be regulated in immature vertebrate testis, such as miR-101, miR-137, miR-92b, miR-18a, and miR-20a. Additionally, miRNAs reported for the first time to be regulated in the testis were also identified, including miR-new289, miR-30c, miR-724, miR-26b, miR-new271, miR-217, miR-216a, miR-135a, miR-new194, and the novel predicted n268. During the transition into puberty stage, distinct expression patterns of miRNAs were observed, some of which were already linked to this process (let-7a/b/c), and others were newly associated with it (miR-15c, miR-2184, miR-145, and the novel predicted n7a and n7b). During the pubertal stage along with the previously discovered miRNAs (miR-20a, miR-25, miR-181a, miR-202, let-7c/d/a, miR-125b, miR-222a/b, miR-190a), new connections of miRNAs (miR-2188, miR-144, miR-731, miR-8157, and the novel n2) were found with the initiation of puberty [29].

Proteomics involves the study of the entire complement of proteins expressed by an organism. It allows researchers to analyze changes in protein expression and post-translational modifications during puberty in fish. This information complements genomics data and provides insights into the functional aspects of the genes involved.

Western blot, Immunohistochemistry and Immunocytochemistry are a few techniques performed for identification and localization of the protein of interest. An increased expression of kisspeptin in brain and pituitary during pubertal onset time has been confirmed in the European Sea Bass with the help of immunohistochemical detection [30]. In a study done in Chinese Sucker (*Myxocyprinus asiaticus*), the kisspeptin-gonadotropin releasing hormone system was highly expressed at the onset of puberty, indicating it to be an important signalling pathway for triggering the HPG axis during the pubertal onset time [31].

2. Endocrine Manipulations

Biotechnological approaches, such as hormone treatment and gene knockdown techniques, have been used to manipulate the endocrine system of fish. By altering hormone levels or disrupting specific genes involved in puberty regulation, the effects on the timing and progression of puberty can be observed.

In Catla fish, administering nano-encapsulated kisspeptin through injections resulted in an upregulation of reproductive genes (GnRH, LH, and FSH) expression, ultimately triggering the initiation of puberty [13]. In Nile Tilapia, the expression of *kiss2* was notably higher during the immature stage compared to the mature stage, indicating its potential role in gonadal development. Additionally, external administration of kisspeptin led to increased levels of estradiol and testosterone in the blood plasma, along with elevated expression of GnRH and gonadotropins in the brain [32]. In 2020, a study involving Senegalese sole utilized an intramuscular injection of kisspeptin, which led to elevated synthesis and secretion of gonadotropins, along with increased testosterone levels in the plasma [33]. In Chub Mackerel, after giving the treatment of leptin to pituitary cells under *in-vitro* conditions, it influenced the release of gonadotropin, which might be a key signal for onset of puberty [21, 22]. The administration of NKB has a notable impact on zebrafish [28], goldfish [34], and tilapia [35], effectively triggering the secretion and production of luteinizing hormone (LH). This indicates that NKB signalling plays a crucial role in regulating the reproductive axis.

3. Molecular Markers

Biotechnology has enabled the discovery and development of molecular markers associated with puberty in fish. These markers can be used for early detection of puberty onset and to study the genetic basis of variations in pubertal timing among different fish populations.

Kisspeptin, Gonadotropin releasing hormone, Insulin like growth factor-1, Leptin, Neuropeptide Y, Neurokinin B and miRNAs, these are few molecular markers identified during pubertal onset in fishes with the help of the biotechnological advancements. Apart from these there are other markers like Makorin ring finger protein 3 (MKRN3), which was the first gene that was identified to have inhibitory effect on GnRH. The decrease in hypothalamic *mkrn3* expression observed in mice during prepubertal development, as well as the loss of MKRN3 function in patients with precocious puberty, provides compelling evidence supporting the role of MKRN3 in repressing the onset of puberty [36]. Next is Delta-like non-canonical notch ligand 1 (DLK1) whose would result in an increase in the formation of kisspeptin neurons. A study shows that female mice deficient in DLK1 experience pubertal onset [37]. However, the regulation of these two molecules (MKRN3 and DLK1) have yet not been studied in a fish model. This arouses an interest to further investigate the effects of them in a fish model. Then hormonal regulators like Melatonin and Serotonin have also been observed to have an indirect regulation on the pubertal onset. The results from the Zebrafish study indicate that melatonin, through receptor-mediated action at the brain level, could potentially stimulate the release of hypothalamic GnRH. This mechanism likely involves the kiss1r system in the GnRH neurons [38]. In Orange-spotted grouper (*Epinephelus coioides*), the melatonin receptor, which shows higher expression at night and lower expression during the day, was down-regulated. This down-regulation led to the up-regulation of kiss2 and GnRH, which exhibit higher expression during the day and lower expression at night [39]. On the other hand, studies done on serotonin suggest that it’s receptors co-expresses with the GnRH neurons and promotes gonadotropin secretion [40-46]. Further investigation is required to properly understand the mechanism behind the pubertal onset and the interaction between all these molecular markers into it.

4. CRISPR/Cas9

The advent of genome editing, such as this revolutionary clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein (Cas9) technology, presents exciting opportunities to expedite sustainable genetic advancements in fisheries and aquaculture. This technology facilitates the investigation of specific genes involved in puberty and helps researchers understand their roles. Its applications benefit from certain attributes of aquaculture species, such as high fecundity, external fertilization, well-established breeding methods, and efficient larval rearing techniques [47].

The use of CRISPR/Cas9 genome editing enables precise, targeted modifications to the genome of the target species, often involving minor changes. Importantly, this technology does not introduce any foreign DNA, addressing significant public concerns about the safety of genetically modified organisms (GMOs) [47]. In recent times, researchers have extensively investigated the CRISPR/Cas9 system to study gene functions and breeding *in vivo* and/or in cell lines of numerous prominent aquaculture species, encompassing over 20 different species, namely Nile Tilapia, Rainbow Trout, Atlantic Salmon, Common Carp, Rohu, and other species [48, 49]. In genome-editing experiments conducted on cultured fishes, the primary focus has been on targeting key genes such as myostatin (*mstn*), which plays a critical role in regulating skeletal muscle growth and fertility, along with sex-related genes like dead end (*dnd*) and doublesex- and mab-3-related transcription factor (*dmrt*) [50].

By targeting and disabling the *mstn* gene in various fishes like Common Carp [51], Red Sea Bream [52], Channel Catfish [53], the outcome has been notable, leading to a significant increase in skeletal muscle mass (up to 16%), shortened body length, improved feed efficiency, and ultimately promoting better overall growth in the fish. In another study done in the Tiger Puffer (*Takifugu rubripe*), CRISPR is utilized to modify growth-associated traits by disrupting the leptin receptor gene, which regulates appetite. This genetic alteration causes the fish to exhibit increased food consumption [54]. The Atlantic salmon, upon the knockout of the germ cell-specific candidate, dead end (*dnd*) gene, exhibited a notable outcome: they remained in an immature state and did not undergo puberty [55]. In a recent development, CRISPR/Cas9 technology was used to generate an all-female population of Common Carp by disrupting the Cytochrome P450 17A1 (*cyp17a1*) gene [56].

Studies with respect to puberty with the help of CRISPR/Cas9 techniques in fishes are scarce. Compared to other genome editing technologies, CRISPR/Cas is more cost-effective, simpler to use, and offers higher precision. As a result, it can be harnessed as an innovative breeding technology in fisheries and aquaculture to address wide-ranging challenges effectively.

In conclusion, by combining these biotechnological advancements with traditional physiological and behavioral studies, researchers are making significant strides in unraveling the complexities of puberty in fish. These insights have implications not only for fish biology and aquaculture but also for our understanding of reproductive processes in other vertebrates, including humans.

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