Chapter 1: Introduction

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1.1. Melatonin

Melatonin is chemically known as *N*-acetyl-5-methoxytryptamine **(Figure 1.1)**. The molecular weight is 232.29 g/mol, the melting temperature is 116.5–118°C, and the half-life is about 45 min (Al-Omary, 2013). Its HPLC stability in aqueous solutions at room temperature, 4°C, and -70°C is up to 6 months (Cavallo and Hassan, 1995). Metabolism of this molecule occurs primarily in the liver and is inactivated by 6-hydroxylation, followed by (mainly) sulfate and glucuronide conjugation. Its main urinary metabolite is 6-sulfatoxymelatonin (Al-Omary, 2013).

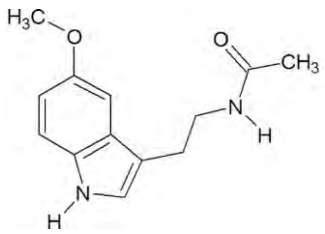

***Figure 1.1**: Structure of melatonin* (Wakahara *et al*., 1972)

1.1.1. Discovery

Melatonin was first isolated from the bovine pineal gland by an American dermatologist, Dr. Aaron Bunsen Lerner, and his group in 1958 (Lerner *et al*., 1958). They introduced the term 'melatonin' for this purified pineal substance in recognition of a previous report that treatment with crude acetone extract of bovine pineal glands to tadpoles of *Rana pipens* caused lightening (blanching) of their skins (i.e., melanophore-contracting hormone; Greek: μελαζ = black; τονζ = tension, in the sense of contraction), resulting in clear visibility of the larger viscera through the dorsal body wall (McCord and Allen, 1917). In the last 65 years, the molecule has received vast attention from researchers and is considered a potent molecule due to its tremendous effect on various physiological functions (Samanta, 2022).

1.1.2. Biosynthesis

Melatonin is synthesized from the essential amino acid L-tryptophan, which undergoes four sequential steps **(Figure 1.2)**. In the first step, L-tryptophan is converted to 5-hydroxy-tryptophan (5-HTP) through the action of Trp-5-mono-oxygenase/hydroxylase within the mitochondria (Lovenberg *et al*., 1967). In the second step, 5-HTP undergoes decarboxylation facilitated by aromatic amino acid decarboxylase, forming 5-hydroxytryptamine (5-HT/serotonin). This enzymatic process occurs within the cytosol (Lovenberg *et al*., 1962). In the third step, 5-HT is acetylated into *N*-acetyl serotonin by the rate-determining enzyme arylalkylamine-*N*-acetyltransferase (AANAT) (Voisin *et al*., 1984). In the final stage, *N*-acetylserotonin is catalyzed by *N*-acetylserotonin *O*-methyltransferase (ASMT), alternatively known as hydroxy Indole-*O*-methyltransferase (HIOMT), which results in the production of *N*-acetyl-5-methoxytryptamine or popularly known as melatonin (Axelrod and Weissbach, 1960).

1.2. Chrono biological features of circulatory melatonin

Circulatory and pineal melatonin exhibited parallel rhythmic changes in a 24-hour light-dark cycle with a peak at night and nadir in the day across all vertebrates. Overall, three types of melatonin rhythms, viz. (i) Type-A, (ii) Type-B, and (iii) Type-C, have been found in various vertebrates, including fish (Falcón *et al*., 2010; Migaud *et al*., 2010). The Type-A profiles are characterized by a discrete peak in the late dark phase, e.g., Atlantic cod, *Gadus morhua* (Porter *et al*., 2000); haddock, *Melanogrammus aeglefinus* (Davie *et al*., 2007). In contrast, Type-B profiles are characterized by a discrete peak in the mid-dark phase, e.g., Nile tilapia, *Oreochromis niloticus niloticus* (Martinez-Chavez *et al*., 2008). However, Type-C is characterized by a rapid rise in melatonin immediately following the onset of the dark period, e.g., Atlantic salmon, *Salmo salar*; rainbow trout, *Oncorhynchus mykiss;* Atlantic halibut, *Hippoglossus hippoglossus;* and most teleosts

(Falcón *et al*., 2010; Migaud *et al*., 2010). Though the aetiology of these different types of melatonin profiles is far from being clearly understood, it might be linked to the ability or not to anticipate photic signals under the control of circadian clocks (Martinez-Chavez *et al*., 2008). However, the study on carp, *Catla catla,* revealed for the first time that the nocturnal peak pattern of serum (Maitra *et al*., 2005), as well as pineal (Mukherjee *et al*., 2014a) melatonin in the same species, may vary from Type-A (during the months of February-March) to Type-B (in the remaining part of the year) with different seasons of an annual reproductive cycle.

1.2.1. Diurnal pattern

Melatonin synthesis in most teleost species was found to be rhythmic in the pineal and serum, and this rhythm is synchronized to the 24-hour light-dark cycle (Seth and Maitra, 2011; Falcón *et al*., 1987; Chattoraj *et al*., 2009). The pineal clock system and rhythmic melatonin synthesis are connected (Appelbaum *et al*., 2006; Vazquez *et al*., 2024) with the expression of clock genes-associated transcription factors, which can activate the *aanat* genes expression, thereby AANAT protein function with the onset of darkness. The photic impulse of the subsequent day resets the clock genes, which drop AANAT enzyme activity and melatonin synthesis in pineal (Ziv *et al*., 2007). The same mechanism is also seen in carp (Mazur *et al*., 2023). In contrast, the salmonids, like rainbow trout (*Oncorhynchus mykiss*), masu salmon (*Oncorhynchus masou*), and sockeye salmon (*Oncorhynchus nerka*), are exceptions, who do not possess the circadian regulation of melatonin production (Zachmann *et al*., 1992; Thibault *et al*., 1993; Iigo *et al*., 2007). Moreover, melatonin secretion and its peak in fish depends on the depth of the water, water temperature (Coon *et al*., 1999; Falcón, 1999), time of the day (dawn and dusk), weather, moon phase (Lopes *et al*., 2023), or latitude, intensity, and spectrum of the light (Falcón *et al*., 2010; Sánchez-Vázquez *et al*., 2019; Vazquez *et al*., 2024).

1.2.2. Seasonal pattern

The variation in melatonin's seasonal rhythms depends on various environmental factors, among which day length or photoperiod is the most crucial as the length of a day is determined by planetary movement, which varies seasonally (Haldar *et al*., 2011). The pattern of hormone concentration serves as an endocrine calendar for seasonal animal breeders because of the photoperiodic regulation of the melatonin rhythm (Haldar and Saxena, 1988). A remarkable negative correlation was found between the seasonal levels of melatonin in pineal and serum and photoperiod, which indicates that melatonin titer is inversely proportional to the duration of light (Porter *et al*., 1999). The daily maximum titers of melatonin in serum and the density of pineal AANAT protein in the late dark phase of March and at midnight in the remaining annual cycle stages were found in carp *Catla catla* (Mukherjee *et al*., 2014a). The highest amplitude of serum and pineal melatonin was found in December and the lowest in September (Mukherjee *et al*., 2014a). Additionally, another work depicts that a decrease in the seasonal light phase can cause a gradual increase in plasma melatonin concentration and *vice versa* in pike perch (*Sander lucioperca)* (Baekelandt *et al*., 2020). Moreover, plasma melatonin level is also influenced by the intensity and wavelength of light as lower intensity (below ~5 lux) of mainly red light pulses (wavelength ~610-687nm) can allow melatonin synthesis (Carazo *et al*., 2013). In contrast, the higher intensity light (above ~5 lux) and also more adversely the blue to green light (wavelength ~437-575) ceases the production of melatonin by the pineal organ (Bayarri *et al*., 2002; Oliveira *et al*., 2007; Brüning *et al*., 2016). Moreover, ocular melatonin also decreases in blue light, suggesting that blue light-sensitive opsin plays a role in suppressing melatonin synthesis in the eye of damselfish (*Chrysiptera cyanea*) (Takeuchi *et al*., 2014). Incoming solar radiation, or insolation, refers to the energy the earth's surface receives through short waves. The length of the day varies by

location and season, directly impacting the amount of insolation received. Long-day result in greater insolation, while short-day lead to less insolation, which can be reflected in higher levels of melatonin (Reiter, 1991) and its receptor expression during the short-day compared to long-day (Lahiri and Haldar, 2009; Haldar *et al*., 2011; Falcón *et al*., 2021).

1.3. Ovarian development in fish and the role of melatonin

Depending upon the reproductive schedule, fish are classified into semelparous and iteroparous species (Hughes and Simons, 2014). Fish species that are characterized by a single reproductive episode before death are known as semelparous (e.g., Anguillid eels and Pacific salmon). On the other hand, species that spawn multiple times over their lifetime are known as iteroparous (e.g., *Catla catla*, Salmonids except for Pacific salmon). Again, based on their spawning habits, fish ovaries may be in synchronous, group-synchronous, or asynchronous conditions. Within the ovary, if only one batch of developing oocytes is found during the reproductive season, it is said to be synchronous (e.g., *Oncorhynchus*); if two distinct groups of oocytes are present at the mature stage of ovary can be considered under group-synchronous development (e.g., *Fundulus heteroclitus*); again if different kinds of oocytes are available in the ovary at the time of spawning those are termed as an asynchronous development, which is exhibited by multiple-spawning fish species (iteroparous) (Shimizu, 2003). Multiple-spawning activity with asynchronous ovarian development can also happen within a single season, which is the most common strategy in teleost.

Seasonal variations in temperature, photoperiod, and food sources determine the course of life for most organisms in temperate and longer latitude regions, including fish. Control systems have, therefore, been developed to allow developmental events like smoltification and reproduction to be synchronized with seasonal variations in the external environment. This guarantees that fish spawning and maturation occur at the

right times of the year to maximize the species' survivability rate. The photoperiod/day length was found to be the most crucial environmental factor that was identified as a potent zeitgeber for fish reproduction and development to synchronize with the season in teleost, including carps (Duston and Bromage, 1986; Whitehead *et al*., 1978; Akhoundian *et al*., 2020). Apart from photoperiod, successful fish reproduction can be performed in tropical and sub-tropical regions under a favourable temperature range and sufficient amount of rainfall (De Vlaming, 1972; Vasal and Sundararaj, 1976; Pankhurst and Porter, 2003), that is generally restricted only during the monsoon seasons. Therefore, for fish farmers, the supplies of eggs, fry, and fingerlings only remain available during the year in subtropical regions (Duston and Bromage, 1986). Seasonality is the major disadvantage in this regard. As reproductive and developmental events depend on photoperiodical cues, it is possible to alter the timing of maturation and spawning of fish species to maintain the supply of its eggs and fry according to commercial demands. However, such manipulation of photoperiod is critical. Based on the research work performed in more than the last six decades, it can be argued that melatonin can be a potent photoperiodic molecule as it can modulate various physiological functions (Talpur *et al*., 2018), including ovarian steroidogenesis (Adriaens *et al*., 2006; Zhao *et al*., 2024), folliculogenesis (Carnevali *et al*., 2011), oocyte maturation (Shi *et al*., 2009; Tamura *et al*., 2012; Maitra *et al*., 2013), and ovulation (Ogiwara and Takahashi, 2016). Melatonin is known to exert its effects not only via different types of G protein-coupled melatonin receptors (Dubocovich *et al*., 2010) but also by penetrating the cell membrane due to its lipophilic nature (Tamura *et al*., 2009; Cruz *et al*., 2014).

1.3.1. Melatonin and ovarian growth

Previous studies in vertebrates confirmed that the effects of exogenous melatonin administration on reproductive function are photoperiod, dose, duration, and time-

dependent (Wang *et al*., 2023; Kim *et al*., 2024). There are sufficient reports that a combination of a long photoperiod and relatively high temperature effectively accelerated the development of the gonads (Garg and Jain, 1985; Srivastava and Singh, 1991). Whereas under long photoperiod, when melatonin titer in the plasma remains low, if the daily intraperitoneal injection of melatonin was administered, reduced ovarian development was noted in a variety of fish species (Fenwick, 1970; Saxena and Anand, 1977; Ghosh and Nath, 2005; Badruzzaman *et al*., 2021). The same inhibitory response of melatonin on gonadal functions was also noted in several subtropical fish (Joy and Agha, 1991) under short photoperiods when the melatonin titter in the plasma remains high. Further, feeding with melatonin-containing pellets for 1 to 2 weeks of tropical damselfish (*Chrysiptera cyanea)* resulted in the induction of atresia and the arrest of recruitment in vitellogenic oocytes in the ovaries (Badruzzaman *et al*., 2013; Imamura *et al*., 2022). This suggests that applying melatonin with fish feed can inhibit gonadal maturity during spawning. On the other hand, a recent report demonstrated that combining oocyte developer hormones with melatonin in fish feed can enhance spawning frequency, spawning time, and egg production in a dose-dependent manner in clownfish (*Amphiprion sp.*) (Arfah *et al*., 2024). A report states that if fish (*Danio rerio*) are kept under long photoperiod but in melatonin-containing water during the daytime, that also can stimulate ovarian development (Carnevali *et al*., 2011). Again, a report on carp indicated that melatonin administration during the early part of the annual reproductive cycle may increase ovarian growth. Thereby, the effect of melatonin on ovarian development varies depending on the species, reproductive status, and mode of administration. Thus, melatonin may exhibit pro-gonadal and anti-gonadal responses (Dey *et al*., 2005; Mondal *et al*., 2017) on ovarian development. Moreover, some reports indicate melatonin can influence the synthesis and release of key reproductive hormones

from the hypothalamus: (i) gonadotropin-releasing hormone (GnRH), (ii) kisspeptins (Dan *et al*., 2024), (iii) gonadotropin-inhibitory hormone (GnIH) (Falcón *et al*., 2007; Takahashi and Ogiwara, 2021), and thereby the reproduction of animals.

Seasonal variations in serum estradiol-17β levels have been reported for many fish species. Whether they spawn once a year or several times during the breeding season, female fish show increased serum levels of estradiol-17β during the rapid growth period of oocytes (Cochrane and Deeley, 1988; Greeley *et al*., 1988; Lamba *et al*., 1983). Melatonin may influence the ovary's production of estradiol-17β as seasonal variations in plasma estradiol-17β levels are negatively correlated with the plasma melatonin levels, which gradually reduces with an increase in day length (Vasal and Sundararaj, 1976). Plasma estradiol-17β levels increased in the long photoperiodical conditions, which indicates melatonin's suppressive influence over the synthesis of estradiol-17β in fish (Choi *et al*., 2016). Under the stimulation of estradiol-17β, yolk globules get deposited in the oocyte's cytoplasm, a characteristic of the vitellogenic phase (Lubzens *et al*., 2010). The liver produces the complex phospholipoglycoprotein called vitellogenin, which is integrated into the oocyte and then processed by proteases to produce lipovitellin with varying molecular weights. This lipovitellin is then stored in yolk globules (Finn, 2007; Carnevali *et al*., 2006; Lubzens *et al*., 2010). Most teleost species have demonstrated that melatonin contributes to the synthesis of vitellogenin in the liver and vitellogenesis in developing ovarian follicles; however, it is assumed that the HPG axis mediates many of these effects (Takahashi and Ogiwara, 2021). According to one report (Panchal and Rani, 2019), melatonin treatment by intraperitoneal route during the pre-spawning period inhibited vitellogenesis and promoted follicular atresia. Moreover, orally treated melatonin could lower the gonado-somatic index (GSI), decrease the amount of vitellogenin in oocytes, and suppress serotonergic activity in fish during the spawning

season (Badruzzaman *et al*., 2020). In contrast, there is evidence of a stimulatory role of melatonin on fish reproduction where greater numbers of classes III (vitellogenic follicles) and IV (maturational follicles) follicles with increased luteinizing hormone receptor mRNA expression and plasma vitellogenin were noted in oocytes treated with melatonin in different doses (Giorgini *et al*., 2012). Steroidogenesis (production of estrogens), which is necessary for oocyte development, produces free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Valenzuela *et al*., 2015), which can hasten oocyte ageing and result in the production of low-quality seeds (Olcese, 2020). On the other hand, melatonin scavenges free radicals (Tan *et al*., 1993), minimizing damage caused by them and enhancing oocyte quality (Reiter *et al*., 2013). The ovarian malondialdehyde (MDA) level in carp varies seasonally, being very high during the post-spawning season and low during the spawning phase of an annual cycle. This pattern negatively correlates with the ovarian melatonin level (Hasan *et al*., 2014), suggesting that this molecule reduces the generated ovarian oxidative stress by enhancing the activities of SOD, CAT, and GPx during oocyte growth and maturation (Maitra and Hasan, 2016; Hasan *et al*., 2014). Nevertheless, no study has examined how melatonin directly affects the synthesis of estradiol-17β and, thereby, the vitellogenic activity in teleost ovaries.

1.3.2. Melatonin and ovarian maturation

Within the ovarian follicles, the growth of the oocyte continues due to the accumulation of yolk materials, but the cell cycle remains arrested in the prophase stage of meiosis I. LH surge is the crucial factor for oocyte maturation, marked by germinal vesicle breakdown (GVBD) - a hallmark of oocyte maturation (Nagahama and Yamashita, 2008). The release of a polar body from the oocyte marks the completion of oocyte maturation and the point at which the oocyte enters a second arrest during meiosis II metaphase

(Nagahama and Yamashita, 2008). The maturation of the oocyte is dependent mainly on gonadotropin (LH), a maturation-inducing hormone (MIH), and a maturation-promoting factor (MPF) (Nagahama, 1997). MIH activates the signal cascade pathway after binding with its receptor at the oocyte surface, ultimately activating MPF (maturation-promoting factor), leading to germinal (nuclear) vesicle breakdown (GVBD). MPF stimulates various intra-oocyte mechanisms, such as chromosome condensation, spindle formation, GVBD, release of the first polar body, and completion of meiosis I (Das *et al*., 2017; El Mohajer *et al*., 2022). In this context, existing research exhibited that the receptor-mediated action of melatonin (Chattoraj *et al*., 2009b; Sakai *et al*., 2019) can promote or accelerate the action of MIH during the process of maturation of oocytes by early formation of MPF (Carnevali *et al*., 2011; Lombardo *et al*., 2012; Kim *et al*., 2023) by reducing cellular oxidative stress and inhibiting apoptosis of oocytes by the action of the receptor-independent function of melatonin, which leads to quality enhancement of the oocytes (Zhang *et al*., 2023). Further, it has been observed that melatonin-pre-treated brooders can produce a higher percentage of matured oocytes when induced with ovaprim (a combination of a salmon gonadotropin-releasing hormone analogue and a dopamine antagonist - Domperidone) – a process generally performed in induced breeding (Moniruzzaman *et al*., 2016). So, melatonin can promote MIH action and enhance the potentiality of an ovulating agent to facilitate reproduction.

1.4. Melatonin in the regulation of fecundity and development of fish embryo

Reports indicated that melatonin may increase the fecundity, hatching rate, and survival of fish embryos in a dose-dependent manner (Lombardo *et al*., 2012, 2014). On the contrary, a report by Kim *et al*. (2018) stated that melatonin application via the oral route (using melatonin-rich pellets) might lead to a decrease in spawning frequency, number of spawned eggs, and gonado-somatic Index (GSI). Again, one report on zebrafish depicted

that the melatonin-pretreated embryo grows faster (Danilova *et al*., 2004) and may protect the embryo from the adverse effects of certain toxic substances like fluorene-9-bisphenol (Mi *et al*., 2019), cadmium contamination (Drąg-Kozak *et al*., 2018). Therefore, most of the existing literature, except a few, indicated that melatonin also significantly influences the process of embryonic development.

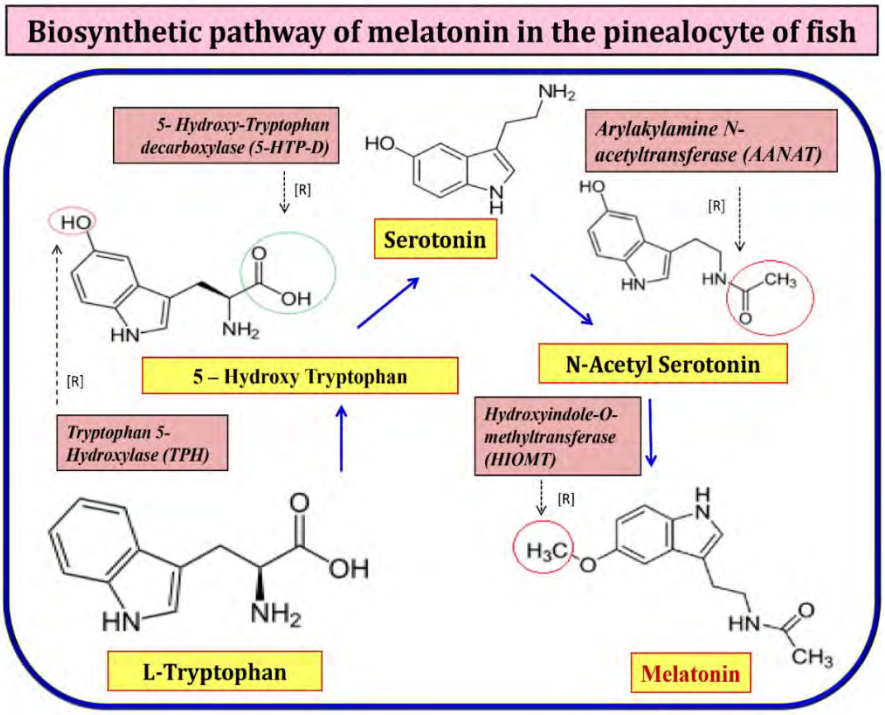

Figure 1.2*: Diagrammatic representation of melatonin biosynthesis pathway within the pinealocyte (represented by the blue box) from the L-tryptophan by four consecutive steps in fish. Product/substrates are within yellow boxes, and enzymes are inside the pink boxes.*