***Review Article***

**Molecular Basis of 20E Biosynthesis and its Function**

**ABSTRACT**

20-Hydroxyecdysone (20E), a crucial steroid hormone in insects, plays a multifaceted role in regulating the developmental, physiological and metabolic processes of silkworm, *B. mori*. 20E biosynthesis during embryogenesis and larval stages, emphasizes the importance of the maternal 3-epimerization pathway and key enzymes such as ecdysone oxidase (*BmEO*) and *3DE-3β-reductase*. Functional studies demonstrate that disruption of these genes leads to reduced ecdysone levels, developmental arrest and decreased hatching rates. The glutathione S-transferase gene *noppera-bo* (*nobo-Bm*) is also critical for ecdysteroidogenesis with its loss of function mutation causing larval lethality due to impaired sterol utilization. Beyond development, 20E modulates immune responses by inducing antimicrobial peptides via the Broad-Complex Z2 (*Br-C Z2*) transcription factor and supports genital disk differentiation through the MAPK signaling pathway. Furthermore, the application of a baculovirus-derived EGT gene to suppress 20E levels in transgenic silkworms has been shown to prolong larval feeding, inhibit pupation and significantly enhance cocoon shell ratio, thereby improving silk yield. These findings collectively underscore the central role of 20E in silkworm biology and offer promising avenues for biotechnological innovations in sericulture.

**Key words:** Diapause, silkworm, biosynthesis, maternal, 20-Hydroxyecdysone, ecdysteroids

1. **INTRODUCTION**

Hormones are the chemical messengers secreted by endocrine system. They play crucial role in regulating various physiological processes in insects (Lafont and Koolman1984). Ecdysteroids are the arthropod steroid hormones, where steroid refers to organic compounds having four fused carbon rings that are produced in the body. The steroids which act as hormones are called steroid hormone. They may be from animal origin (zooecdysteroids), plant origin (phytoecdysteroids) or fungi origin (mycoecdysteroids) (Baruah, 2020). Ecdysteroids in insects regulate various activities like development, moulting, metamorphosis and to some extent reproduction. In insects two important ecdysteroidogenic hormones necessary for growth and development are ecdysone(E) and 20-hydroxyecdysone (20E) (Makka, 2002). Ecdysone most commonly known as moulting hormone or alpha-ecdysone (α-Ecdysone) is the prohormone or precursor for the synthesis of its biologically active form 20E. Hydroxylation at carbon-20 yields 20E or beta-ecdysone (β-Ecdysone) which acts on the genes and gene factors responsible for development and metamorphosis. (Baruah, 2020). The 20-hydroxyecdysone (20E) steroid hormone plays critical role in insect development and physiology. It regulates the formation and growth of imaginal discs such as wing and genital discs, that are essential for adult structure formation. Additionally, 20E is involved in controlling of diapause and embryogenesis, ensuring proper developmental transitions (Tawfik *et al.*, 2002). During pupal-adult metamorphosis, 20E facilitates the degradation of larval tissues, making way for adult organ development. Beyond development, 20E also modulates the innate immune response by stimulating the production of various antimicrobial peptides (AMPs). These include lysozymes, which hydrolyze peptidoglycan in bacterial cell walls; gloverins, which inhibit bacterial outer membrane synthesis; and cecropins, which are effective against a broad spectrum of microbes including gram-positive and gram-negative bacteria as well as fungi (Wang *et al.*, 2008).

1. **Biosynthesis pathway of ecdysteroids**

The head of insects consist of neurosecretory cells (NSCs) present in the either half of the brain called intercerebralis. NSCs upon receiving stimulus from the environment or the body in turn stimulate corpora cardiaca to secrete a neuropeptide, prothoracicotropic hormone (PTTH). PTTH act on prothoracic glands and regulate synthesis of ecdysone. The dietry sterols are incorporated into ecdysteroidogenic organs like prothoracic gland and ovaries and undergo series of hydroxylation reactions (Nagasawa *et al.,* 1986, Mizuguchi *et al.,* 1987, Sakurai *et al.,* 1989).



**Fig.1: Ecdysteroids biosynthesis and utilization pathway in insects**

 The ecdysteroids biosynthesis pathway in prothoracic glands is well documented. It starts with conversion of cholesterol to 7‐dehydrocholesterrol (7dC). This step is mediated by *Rieske oxygenase* enzyme, designated Neverland (*Nvd*). Between 7dC and first upstream compound there is highly characteristic ecdysteroid structure, diketol, which lays the vital “black box.” Black box includes series of uncharacterized and unidentified reactions that ultimately result in oxidation of 7dC to diketol. Differential expression in *B. mori* demonstrated involvement of *CYP307A1* (*Spo*) in the black box, which appears to the rate‐limiting enzyme (Marchal *et al.,* 2011). Non‐molting glossy (*Nm‐g* in *B. mori*) was reported to be involved in the initial steps of ecdysteroid biosynthesis. Hydroxylation at C25, C22 and C2 is mediated by *CYP306A1* (*Phm*), *CYP302A1* (*Dib*) and *CYP315A1* (Sad), respectively (Spindler *et al.,* 2017) and the final hydroxylation is catalyzed by fourth hydroxylase, *CYP314A1* (*Shd*) or ecdysone 20‐hydroxylase (*E20OHase*) in from ecdysone to 20‐hydroxyecdysone (20E) in the peripheral target tissues of *B. mori*. Subsequently, 20E binds with the ecdysone receptor (*EcR*), that forms a dimer with Ultraspiracle (*USP*) (Lammerding-Köppel *et al.,* 1998). This complex further binds to ecdysone response element (*EcRE*) and regulate transcription of the downstream target genes. Three levels of genes transcription occurs. First, transcription of early genes, such as *E75*, *E74* and broad-complex (*Br‐C*). Early‐late genes, such as hormone receptor 39 (*HR39*) and 3 (*HR3*), are then expressed (Huet, *et al.,* 1995). Subsequently, these early and early‐late genes induce transcription of late genes, such as fushi tarazu transcription factor 1 (*FTZ‐F1*) (Gu *et al.,* 2021).

1. **20E biosynthesis during embryonic stage and larval development**

Ecdysteroids are mostly synthesized from prothoracic glands (PGs). However, studies have found the early embryonic development in insects was regulated independently by PGs (Rees, 1995). In addition, embryonic active ecdysteroids are present before PGs formation indicating that those ecdysteroids are of maternal origin and titre of which is important for the normal development of embryo, suggesting that there must be redundancy in synthesis of ecdysteroids in mother moth (Rubenstein, 1982). Therefore, various pathways are mediating synthesis of maternal ecdysteroids, of which 3-epimerisation pathway is among the important pathway (Hoffmann and Lagueux, 1985).

 20E is firstly converted into 3-dehydroecdysone (*3D20E* or *3DE*) by gene ecdysone oxidase (*EO*) then *3DE-3α-reductase* catalyze 3DE to 3-epiecdysone. This intermediate product, 3DE (or 3D20E) also be reduced reversibly to active ecdysone by *3DE-3β-reductase.* Previous studies shown that most of the lepidopteran insects can store large amount of 3DE in their prothoracic glands at immature stages (Kiriishi, 1990). When insects need, 3DE is released and reduced to ecdysone rapidly. The function of the silkworm *BmEO* and *3DE-3β-reductase* and pathway involvedin strain N4 was demonstrated how the silkworm utilizes this pathway to regulate the ecdysone titer of the embryo (Milner, 1985).

Temporal expression patterns of genes involved in pathway showed that, on day one of pupal stage, the expression of *BmEO* gene was detected. The transcription level gradually increased with the development and reached peak at 8th Day after pupation, when ecdysone concentration became almost undetectable indicating that *BmEO* may play key role in converting ecdysone to 3DE during pupal stage. However, 3DE-3α-reductase gene level was low throughout the pupal stage (Wang *et al*., 2018).

The spatial expression profiles of *BmEO* gene are different in male or female tissues of the pupa at the 7th Day (Kiguchi *et al.,* 1981). The transcription signals of the gene were detected in fat body, hemocyte, gonad and weak signals in the pupal head. Furthermore, no sex bias was observed for *BmEO* gene expression in fat body, hemocyte and head (Richards *et al.,* 1987). However, *BmEO* gene was predominantly expressed in the female ovary and the expression level of the gene in ovary was almost 10 times higher than that in testis. The expression divergence of *BmEO* gene between sexes indicates that the gene might take part in female biological process (Hanaoka and Ohnishi 1974). Overall, the *3DE-3β-reductase* gene had much higher expression level than other two genes (*EO* and *3DE-3α-reductase*) and it was expressed from 1st Day after oviposition and reached a peak at 3rd Day after oviposition. Then, the expression level decreased gradually to the basal level as the embryonic development continued. Interestingly, the gene is just expressed ahead of the increase of ecdysone titer. This timing ensures conversion of stored 3DE into active ecdysone right when embryos need it for key processes like segmentation and organogenesis (Yamada and Sonobe 2003, Shen *et al.,* 2018).

1. **The function of *BmEO* gene during the pupal stage of the silkworm**

*BmEO* gene was prominently expressed in the pupal ovary. Knocking-down of mRNAs levels of *BmEO* gene (Day 7 of pupa) showed phenotypic variation examined between control and RNAi pupa (Fakuda *et al*., 1940). *BmEO*-dsRNA injected silkworms grew and developed normally like EGFP-dsRNA injected silkworms. *EO* can convert ecdysone into 3DE. Therefore, the fluctuation of 3DE was examined in the ovary of the female silkworm after RNAi experiment. The concentration of the 3DE extracted from RNAi silkworm was significantly reduced, indicating *EO* could take part in the accumulation of 3DE in the silkworm eggs (Tsuchida *et al.,* 1987).

In addition, the development of eggs laid by *EO* RNAi moths was arrested at organogenesis stage. Additionally, the product of *EO* is 3DE which also can be synthesized into ecdysone (Okamoto *et al*., 2007). Thus, 3DE was injected to rescue the *BmEO* RNAi eggs. The newly laid eggs of the *BmEO*-RNAi adults were collected and then 3DE was injected into the eggs. Injection with 3DE significantly elevated the hatching rate. Meanwhile, injection with 3DE could increase the 20E level at the 2nd Day after treatment. Taken together, the results indicated that maternal *BmEO* is important for the biosynthesis of 20E during the embryonic development (Takeuchi *et al.,* 2000, Kiguchi *et al.,* 1981, Sonobe *et al.,* 2001)

1. **The function of *3DE-3β-reductase* gene during the embryonic development**

3DE can be directly converted into ecdysone by *3DE-3β-reductase*. Two days after RNAi injection, the transcription level of the gene significantly decreased. Interestingly, similar to the maternal *BmEO* RNAi experiments the hatching rate of the eggs injected with *3DE-3β-reductase* dsRNA was also significantly lower. In addition, the down-regulated expression of the *3DE-3β-reductase* gene resulted in the significantly declined 20E level compared with the control at embryonic stages.

Furthermore, the decreased 20E titer significantly affect the normal development of embryos. Development of about 75% embryos after RNAi treatment was arrested at the stage 20 (segmentation of head and thorax) or stage 21 (early blastokinesis). The stages were determined based on a previous study (Miya, 1984). Furthermore, 20E treatment could rescue the abnormal development. Taken together the results revealed that ecdysone 3-epimerization pathway might have been involved in the synthesis of molting hormone during embryonic development of the silkworm (Weirich *et al*., 1993).

1. **Role of *glutathione S-transferase gene* *noppera-bo***

*Noppera-bo* (*nobo*)is a gene involved in incorporation of dietary sterols form food to ecdysteroidogenic tissue and utilization of cholesterol metabolite product (Nagata *et al.,* 1987, 1992). *nobo* gene encodes glutathione S-transferase (*GSTs*). *GSTs* catalyse the conjugation of glutathione to a wide variety of endogenous and exogenous electrophilic compounds thereby protecting macromolecules from oxidative stress (Marchal *et al.,* 2012). Phylogenetic analysis suggests that GST genes belonging to the *nobo* family are also found in other dipteran and lepidopteran species; like *GSTe8* in the mosquito Anopheles gambiae and GSTe7 in the silkworm *B. mori* (Yu *et al.,* 2008). To avoid using a confusing numbering system to represent the orthologous GST genes of different insect species, a unique subfamily name, *noppera-bo* (*nobo*) was proposed, for these orthologs (Enya *et al.,* 2014).

The qRT-PCR experiments revealed that *nobo-Bm* in w1 pnd strain was highly expressed in the PGs and weaker expression in rest other tissues. Temporally, changes in the *nobo-Bm* expression level in the PG closely correlated with changes in the ecdysteroid titer of the hemolymph during 4th and 5th instar larval development which was similar to the expression pattern of other ecdysteroidogenic genes in *B. mori* (Namiki *et al.*, 2005; Ono *et al.*, 2006; Yoshiyama *et al.*, 2006). These results support hypothesis that *nobo-Bm* is involved in the regulation of ecdysteroid biosynthesis in the PG in *B. mori.*

To determine whether the *nobo-Bm* gene plays an essential role in *B. mori* development, a *nobo-Bm* mutant strain was generated using TALEN-mediated gene targeting technology (Cermak *et al.,* 2011). The mRNAs encoding the TALEN pairs were microinjected into the embryos to induce a double-strand break within the 2nd exon. Eventually a *nobo-Bm* mutant strain was isolated that lacked 87 bp and had also exogenously added 2 bp in the 2nd exon and 2nd intron of the wild type *nobo-Bm* locus. This deletion allele was called *nobo-Bm****Δ****85*, lacks the exon - intron junction between the 2nd exon and the 2nd intron. The predicted protein encoded by the *nobo-Bm****Δ****85* allele lacks half of the GST N-terminal domain and the entire GST C-terminal domain, suggesting that the *nobo-Bm****Δ****85* allele is functionally null (Takasu, 2013).

Under regular rearing conditions, *nobo-Bm****Δ****85* homozygous mutants died at the 2nd instar stage; they never grew into the 3rd instar stage or beyond, while wild type and *nobo-Bm****Δ****85* heterozygous animals exhibited no developmental phenotypes. Interestingly, the cuticle of *nobo-Bm****Δ****85* homozygous mutants eventually became glossier than that of the control animals. This glossy cuticle phenotype is reminiscent of the classical *B. mori* mutant non-molting glossy (nmg), which is loss-of-function allele of the ecdysteroidogenic gene nm-g/shroud (Enya *et al*., 2015, Ayres *et al.,* 2013, Nagata *et al.,* 1987).

The sterol levels were examined in the PG cells of *nobo-Bm*D85 homozygotes. Mass spectrometric analysis revealed that the PGs from *nobo-Bm*D85 homozygotes second instar larvae accumulated significantly higher levels of 7dC, a cholesterol metabolite, than the PGs from control animals (wild type and *nobo-Bm*D85 heterozygotes). There were no statistically significant differences in the levels of cholesterol and b-sitosterol between the control and *nobo-Bm*D85 homozygous PG cells. These results suggest that nobo, *nobo-Bm* is essential for development and for the regulation of 7dC utilization in the PGs of *B. mori* (Enya *et al*., 2015, Nagata *et al.,* 1992)*.*

1. **20-Hydroxyecdysone regulates lysozyme transcription through *Br-C Z2* gene**

In insects, the Broad-Complex (*Br-C*) gene regulates the growth and metamorphosis. Most of the *Br-C* isoforms contain two highly conserved domains including a BTB (bric-a-brac, tramtrack and Broad-Complex) domain for protein–protein interaction and a pair of C2H2 zinc fingers. The developmental role of Br-C gene has been well studied in different insect species. In *Drosophila melanogaster* it controls the production of ecdysone-inducible genes (Karim *et al.*, 1993). It stimulates the formation of both wing disc and epidermis in *Manduca sexta* pupae (Zhou *et al.*, 2001). While, in *B. mori Br-C Z2* has been found to induce the expression of WCP10 in wing disc (Ma *et al.,* 2019)

*BmBR-C Z2* was expressed high in the testis, head and hemocytes were higher compared with that in other tissues. 20E was administered into 5th instar silkworm larvae to determine its effect on the expression of *BmBR-C Z2*. Hemocyte samples collected at different time points revealed that *BmBR-C Z2* mRNA level was remarkably high at 3, 6, 12, 24, 48 hours; however, it was greatest after 6 h of 20E administration. Furthermore, approximately similar *BmBR-C Z2* expression patterns were observed in the BmE cells upon 20E treatment suggesting that 20E regulates expression of *BmBR-C Z2.* To determine whether or not the activation of *BmBR-C Z2* can increase the production of AMPs, over-expression of *BmBR-C Z2* in BmE cells was performed using pSL1180-A4-SV40 DsRed-BmBr-C-Z2 vector. Localization of *BmBR-C Z2* protein in the nucleus of the normal cultured and transfected cells. Total RNA analysis revealed that several AMPs expression was up-regulated in the transfected cells compared with the control group. This implies that *BmBR-C Z2* is part of the immune regulatory pathway, possibly preparing the insect for immune challenges during hormonally sensitive periods (like molting, when cuticle defenses are weakened) (Zhang *et al*., 2017).

1. **20E significance in sex expression**

The effects 20E on genital disk growth was evaluated using male disks, because they were bigger in size and therefore easier to collect without damage than female disks. The male genital disk develops into inner and outer sexual organs (Koyama *et al*., 2013 Nijhout and Callier, 2015, Nijhout *et al*., 2007). The upper region in develops into the external genitalia (penis), while the lower region into the internal genitalia (ejaculatory duct, seminal vesicle and accessory gland) (Swevers *et al.,* 2003, Suzuki *et al.*, 2005). During pupal-adult development, the ejaculatory duct and accessory gland elongate and this elongation can be induced *ex vivo* by 20E (Moriyama *et al.,* 2016). Therefore, the length of the disk was used as an index of disk development. 20E induced remarkable elongation of the disks, especially at the presumptive ejaculatory duct and accessory gland. Interestingly, 20E also promoted protein synthesis (Shinbo *et al.,* 1989).

To explore the signaling pathway involved in the induction of disk elongation, the MAPK pathway hypothesized to plays a key role, as this pathway is widely used in the regulation of cell proliferation and differentiation (Elmogy *et al.*, 2006; Manaboon *et al.*, 2009). Thus, when MEK inhibitor, U0126, used, it inhibited both 20E-induced elongation and protein synthesis of the genital disks in a dose-dependent manner, suggesting that 20E induces the development of the genital disk through the MAPK pathway (Fujinaga *et al*., 2017).

1. **20E expression andcocoon trait**

Ecdysteroid UDP glucosyltransferase (EGT) is baculovirus-encoded protein, secreted by alfalfa looper, *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and transfers UDP-glucoside to the hydroxyl group of C-22 in 20E to generate 20E 22-β-D-pyran glucoside (Evans and Reilly 1998, Shikata *et al.,* 1998). The EGT secreted by the virus upon infection can inactivate 20E in the host, thereby hindering normal moulting/eclosion. The GAL4/UAS system, used to express EGT from the LP3 promoter in fat body of last-instar silkworm. The EGT protein secreted into the silkworm haemolymph maintained 20E at low level, allowing to investigate role of 20E in the utilization of available nutrients for silk production. "UAS" stands for "Upstream Activating Sequence," that is a cis-acting regulatory element that is targeted by Gal4 for activating the expression of downstream genes (Chaturvedi *et.al.,* 2011).

Two genetic vectors were developed and inserted into silkworms. When hybrid offspring (E3G and E4G lines) were generated, they showed longer feeding and cocoon-spinning periods, resulting in higher silk output (especially cocoon shell weight and ratio). However, EGT overexpression also blocked metamorphosis, preventing larvae from turning into pupae. In E4G, female larvae mostly failed to spin cocoons and died after feeding, leading to lower cocooning success compared to E3G. Wang *et al.,* (2010) showed that the treatment of 20E decreases the consumption of food in silkworm larvae. ELISA tests showed that 20E levels in E3G/E4G larvae were consistently lower than in normal silkworms, confirming that EGT reduced 20E. E4G silkworms had more EGT and lower 20E than E3G. When 20E was fed to these silkworms, cocooning improved in females, but the overall silk ratio in males dropped. Earlier studies have reported that JH can inhibit 20E secretion during the early days of *B. mori* last (fifth)-instar larvae and further extend the duration of feeding and silk yields (Akai *et al.,* 1985, 1971, Sakurai *et al.,* 1989).

Overall, reducing 20E through EGT expression extended larval feeding time, blocked pupation and increased silk production. This opens up possibilities for developing genetically modified silkworms that produce more silk and don’t need pupal killing, as they never reach the pupal stage (Gu and Chow, 1993). It can be inferred that expression of baculovirus-derived EGT 20E, delay metamorphosis and extend the larval feeding duration and also cocoon-spinning, thereby increasing silk yield. Excessive EGT expression however, especially in females (as seen in E4G), block the cocooning causing death, showing inverse relation between silk production and survival. This strategy genetically offers potential for further development of high yielding, non-pupating silkworm strains, thereby reducing the need for pupal killing in sericulture (Sun *et al.,* 2016).

1. **CONCLUSION**

20-hydroxyecdysone (20E) is a key hormone in *B. mori*, regulating embryonic development, molting, metamorphosis, immunity, sex differentiation and silk production. During early embryogenesis, maternally derived 20E is synthesized via the ecdysone 3-epimerization pathway involving enzymes like ecdysone oxidase and *3DE-3β-reductase*, essential for proper development and hatching. The *noppera-bo* (*nobo-Bm*) gene also supports ecdysteroid biosynthesis by aiding sterol metabolism in prothoracic gland. 20E additionally enhances immune response by inducing antimicrobial peptide genes through the *Br-C Z2* transcription factor and promotes genital disk growth via the MAPK pathway. Importantly, genetic suppression of 20E using a viral *EGT* gene in transgenic silkworms prolongs larval feeding, blocks pupation and increases silk yield especially cocoon shell ratio offering new potential to enhance sericulture productivity and eliminate the need for pupal killing.

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