***Review Article***

**Recent Advances in Elucidating the Mechanism of Embryonic Diapause in Silkworm, *Bombyx mori* L.**

**ABSTRACT**

Diapause in silkworm *B. mori* is a complex adaptive strategy that ensures survival during adverse environmental conditions by halting embryonic development. Diapause hormone (DH), a 24-amino acid neuropeptide secreted by the suboesophageal ganglion of female pupae, plays a central role in inducing embryonic diapause by modulating trehalose metabolism, glycogen accumulation and oxygen consumption. Diapause initiation, maintenance and termination are tightly regulated by temperature, photoperiod and oxygen availability, with mechanisms involving calcineurin signaling, reactive oxygen species (ROS) and ecdysteroid biosynthetic and signaling pathways. Expression patterns of key genes such as those coding for UDP-glycosyltransferases, glucose dehydrogenase and cytochrome P450 enzymes differ significantly between diapause and non-diapause eggs. Artificial techniques such as chilling, acid treatment and novel corona discharge methods offer effective strategies for diapause termination. Understanding these molecular and physiological pathways provides a foundation for targeted manipulation of diapause, with significant implications for improving sericulture practices and silkworm productivity.

**Key words:** Diapause, non-diapause, hormone, enzymatic regulation, calcineurin, corona treatment

1. **INTRODUCTION**

Diapause is an important adaptation in many insect species enabling them to sustain in regions which would otherwise be unfavorable for permanent habitation and to maintain high numbers in an environment which might otherwise support only a low population. The term “diapause” was applied by Wheeler to egg stage of short-winged meadow katydid, *Conocephalus ensiferum* at which its development was ceased (Wheeler, 1893). Later the scope of diapause widened into various stages of insects as “period of arrest in ontogenetic (origin and development of organisms) development” (Chahil *et al.,* 2018). The nature of diapause in mulberry silkworm, *B. mori* is basically determined by manifestation of genetic characters and endocrinological mechanisms under the influence of environmental stimuli, such as temperature and photoperiod (Yokoyama, 2021).

Diapause begins on the second day of oviposition when cephalic lobe and caudal lobe are formed at anterior and posterior region, respectively on embryo and complete embryonic diapause seen after third day (Liang *et al.,* 2018). The percentage of embryonic cells in G1, S and G2 phase of cell division cycle were 10, 35 and 55%, respectively, at the stage of formation of cephalic lobes, whilst 98% of cells were in G2 at diapause stage. After termination of diapause the cells rapidly entered S phase through G1 from M phase indicates that embryonic cells are arrested at G2 stage of cell division. (Nakagaki *et al*., 1991)

Diapause in insects can be categorized based on seasonal variations, developmental stages and environmental influence. According to seasonal variations, diapause may occur during summer (aestivation), as seen in coccinellids, or during winter (hibernation), observed in species like *B. mori* and grasshoppers (Hand *et al*., 2016). Based on life stages, diapause can take place in the egg stage (embryonic diapause) in *B. mori* and *Aedes aegypti*, the larval stage in *Cydia pomonella*, the pupal and egg stage in *Antheraea yamamai* and the adult or reproductive stage in *Musca autumnalis* (Yevgeniya, 2013). In terms of environmental influence, diapause is classified as obligatory or facultative. Obligatory diapause occurs regardless of external conditions, as in univoltine *B. mori* and the cinnabar moth, while facultative diapause is environmentally induced, seen in multivoltine *B. mori* and certain mosquitoes (Hadley, 2025).

1. **Diapause hormone (DH) composition**

DH is the active principle of diapause. It is a neuropeptide hormone synthesized and released by sub-oesophageal ganglion (SG) of female pupae programmed to lay diapausing eggs (Okitsugu, 1996, Imai *et al*., 1991). DH is isolated from SGs and determined to be a 24 amino acid peptide and the sequence follows: Thr-Asp-Met-Lys-Asp-Glu-Ser-Asp-Arg-Gly-A1a-His-Ser-Glu-Arg-Gly-Ala-Leu-Cys-Phe-GIy-Pro-ArgLeu-NH2 (Gu *et al.,* 2020). Two forms of DH (DH-A and DH-B) have been identified which consists of 12 – 14 amino acids. DH-B activity was more than threefold that of DH-A (Yamashita, 1996). Major functions of DH include determines voltinism, inhibit cytochrome oxidase enzyme thereby reducing respiration rate and decreases oxygen consumption due to which metabolism is also reduced (Denlinger, 2005).

1. **Stage dependent changes in diapausing eggs**

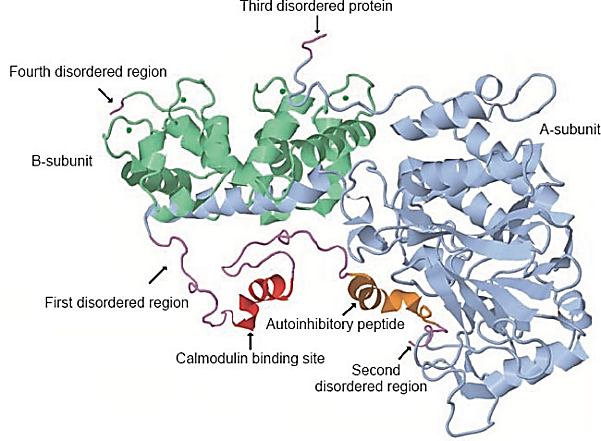
Incubation g *B. mori* eggs as low as 15oC causes production of non-diapause eggs in the next generation, whereas, diapause eggs are induced by incubation at 25 oC. Egg diapause is regulated by photoperiod as well as temperature during embryonic stage of the female and is completely independent of photoperiod during post-embryonic development (Feng *et al*., 2012). Glycogen phosphorylase involved in sorbitol synthesis is observed in induction phase. During diapause initiation, reduced oxygen and water content has been observed. The oxygen uptake is reported to be 30µl/g of eggs/hr. Within 4 hrs of oviposition reached to 100 µl/g of eggs/hr to 24 hrs and thereafter declined rapidly reaching to 10µl/g of eggs/hr on 10th day and 8µl/g of eggs/hr on 70th day. The decrease in oxygen uptake by day 10 at 25°C indicates the establishment of a stable physiological status of diapause (Jiang *et al*., 2017). During diapause termination, during which NAD+ sorbitol dehydrogenase activity increases thereby leading to glycogen synthesis. Exposure of silkworm diapause eggs to oxygen or acid treatment also terminates diapause in eggs (Hiroyoshi *et al.*, 2018).

1. **Termination of Diapause**

Termination of diapause can be done by cold storage – chilling (hibernation schedule) (Clark and Worland 2008). Exposure of diapausing silkworm eggs to a temperature as low as 5°C over 60 days completely terminates diapause and embryogenesis resumes when these eggs are transferred at 25°C. Optimum temperature to break the embryonic diapause is in between 5°C – 7.5 °C. The required chilling duration to break the diapause depends on the time gap the eggs have been kept for aestivation at 25°C after oviposition. Hydrochlorization (hot or cold acid treatment), There are two methods of HCL treatment. In the first method 20 – 24 hrs old oviposited eggs are soaked in HCL solution (specific gravity: 1.075 at 15°C) at 46.1°C for 5 minutes. This avoids the eggs to enter into diapause and hence when incubated at 25°C, larvae hatch in 10 -11 days after treatment. In the second method 20 – 24 hrs old oviposited eggs are soaked in HCl solution (specific gravity 1.10 at 10°C) at room temperature for 60 – 90 minutes (Gong *et al*., 2016, Dandin and Giridhar, 2014). Cold storage and hydrochlorization (chilling and acid treatment) method, 48 hrs old eggs are first chilled at 5oC for more than 30 days and then soaked in HCI solution (specific gravity 1.10 at 15oC) at 48oC for 5 minutes. This treatment causes diapause eggs to hatch within two months after oviposition. (Singh *et al*., 2013)

1. **Physiological and biochemical changes during diapause**

Diapause eggs showed high accumulation of 3-hydroxykynurenin and glycogen. Dramatic changes exist on the commencement of diapause, where 3-hydroxykynurenine from the haemolymph in the developing eggs is oxidized to ommochrome, resulting in the dark coloration of the diapause destined eggs and glycogen is mainly converted into sorbitol, acts as an anti-freeze for the diapause embryo (Zhang, 2017). DH acts directly to induce expression of trehalase gene in the developing ovary and enhances trehalase activity localized in plasma membrane of the vitellogenic follicles (Deng *et al*., 2018). In the oocyte, glucose is immediately utilized to synthesize glycogen as a storage reserve, by which hyperglycogemia is induced in eggs, a pre-requisite for diapause initiation (Singh and Saratchandra, 2002).



**Fig. 1: Structure of Calcineurin (Bo *et al*., 2019)**

1. **Expression of calcineurin in relation to the silkworm embryonic diapause**

Calcineurin (CN) is a Ca2+ /calmodulin-activated serine/threonine protein phosphatase known to be involved in a myriad of cellular processes and signal transduction pathways (Gu *et al*., 2010). It is activated by the influx of intracellular Ca2+. An immunoblot analysis found that CN is a heterodimeric protein consisting of a 59-kDa catalytic A subunit, calcineurin A (CNA) and a 19-kDa regulatory B subunit, calcineurin B (CNB) (Gu *et al*., 2021). The expression levels of CNA, CNB and a CN regulator calcipressin vary in diapausing and non-diapausing eggs (Hsieh *et al*., 2019).

Western blot analysis of *B. mori* eggs showed that on day 4 post-treatment, CNA levels were lower and CNB levels higher in HCl-treated eggs compared to diapause eggs, while CAL levels remained unchanged. Total protein levels (via HSP70) were stable. In HCl-treated eggs, CNA levels decreased and CNB levels increased during early embryogenesis, suggesting a role in diapause regulation. In contrast, diapause eggs maintained high CNA and CAL levels with declining CNB, indicating a possible suppression of CN activity. The high CNB expression and enzymatic activity in developing (non-diapause) eggs suggests that CN is involved in embryonic development, possibly regulated by non-catalytic CNA domains (*viz.* CNAac (CNAa and CBD) and CNAaci (CNAa, CBD and AI) (Denlinger and Armbruster 2014). It was also suggested that calcipressins operate as endogenous feedback inhibitors of CN (Kingsbury and Cunningham, 2000) and over expression experiments in several organisms revealed that calcipressins inhibit CN activity (Liu, 2003). Thus, it was hypothesized that high protein levels of calcipressin detected in diapause eggs may also contribute to very low endogenous CN activity. The subunit CNB was localized in serosa cells and yolk cells, suggesting CNB is activated by intracellular Ca2+ or efflux Ca2+ resulting from HCl treatment and that it plays a role in the molecular mechanisms of artificial diapause prevention or the breaking of diapause in the silkworm (Sato *et al*., 2021). Additional studies to clarify the regulation of CN activity and map upstream and downstream signaling would provide new insights into understanding possible mechanisms regulating embryonic development in *B. mori*.

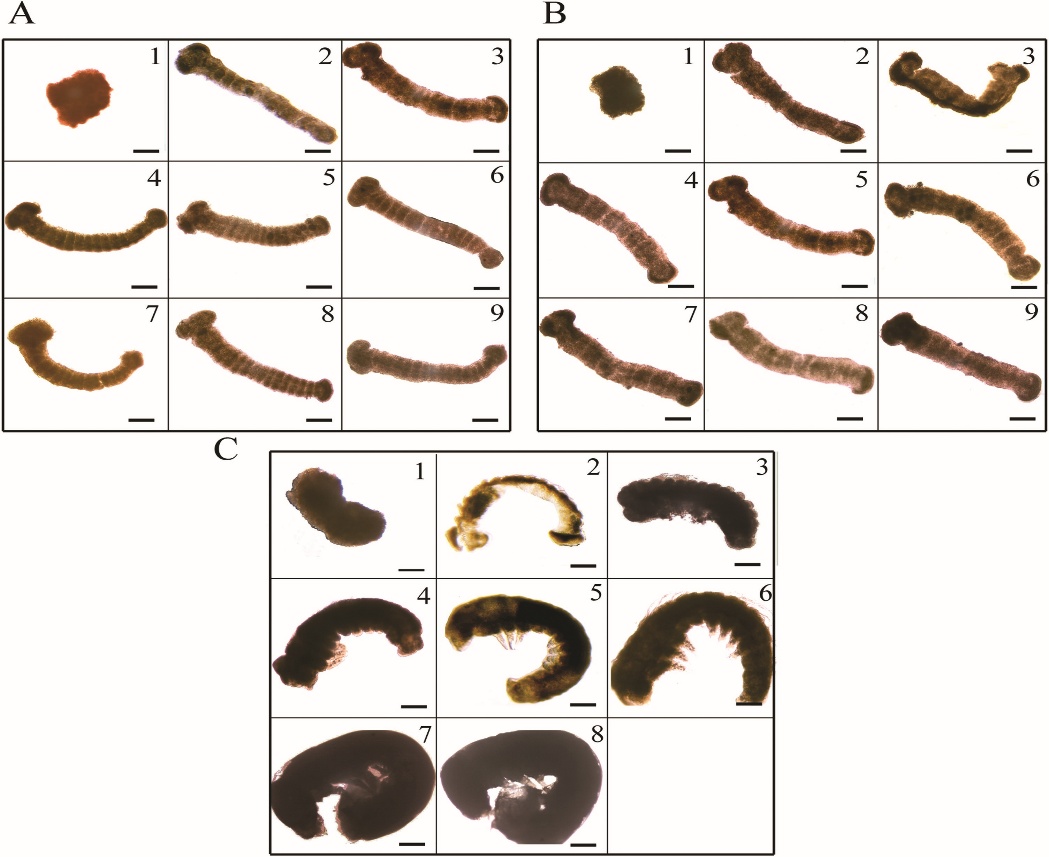
1. **Expression of Kinases and Phosphatases**

Protein kinases C (PKCs) are a family of serine/threonine kinases that are ubiquitously present in animal tissues and play central roles in eukaryotic cellular differentiation, activation of signaling cascades and survival (Tougeron, 2019). PKCs have been classified into three subfamilies: classical (cPKC), novel (nPKC) and atypical (aPKC) (Jing *et al.,* 2018). Western blot analysis of PKC-dependent protein phosphorylation and PKC protein in silkworm eggs showed higher PKC protein levels in HCl-treated eggs compared to control (water treated) eggs. Temporally total PKC protein levels also showed gradual increases during the first 9 days of embryonic development in HCl-treated eggs and gradually decrease in diapausing eggs. Increased phosphorylation levels of multiple PKC-dependent proteins, PKC protein levels and PKC enzymatic activity in developing eggs during the early and middle stages of embryonic development in non-diapause eggs and cold treated eggs (5 °C for 70 days) are likely related to embryonic development (Kostal, 2006). The termination of *B. mori* embryonic diapause under low temperature makes extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling activate in the yolk cells ([Iwata *et al*., 2005](https://www.frontiersin.org/journals/cell-and-developmental-biology/articles/10.3389/fcell.2020.593613/full#B44)).  ERK/MAPK increases sorbitol and 20-hydroyxecdysone (20E) metabolism by regulating the transcription of downstream genes thereby promoting embryonic development, yolk-cell dispersion as well as yolk protein degradation ([Fujiwara *et al*., 2006](https://www.frontiersin.org/journals/cell-and-developmental-biology/articles/10.3389/fcell.2020.593613/full#B26), Fujinaga *et al*., 2017). Adenosine monophosphate (AMP)-activated protein kinase (AMPK) regulates the functions in embryonic development and hatching of *Nilaparvata lugens* (Rice brown plant hopper) (Klee *et al*., 1998). Likewise, phosphotransferase-protein phosphatases are involved in dephosphorylation regulate signal transmission throughout the cell. PP1-C and PP4-C phosphatases undergo differential changes in protein levels during the embryonic stage between diapause and developing eggs. Higher protein levels and enzymatic activities of PP4-C and PP1 are likely related to the embryonic development of *B. mori* (Gu and Linn 2022)*.*

1. **Effect of Hypoxia on embryo development**

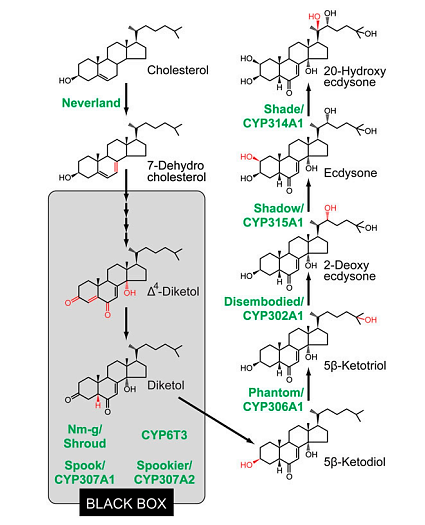
Hypoxia refers to low levels of oxygen in the body tissues. When non-diapause silkworm eggs were exposed to 5% and 10% oxygen concentrations for more than 2 days, their hatching time was significantly delayed compared to control normoxia (25°C, RH 75%) and the eggshell color changed from pale yellow to pale red (5% O2) which could be attributed to deposition of serosal layer (Jing *et al.,* 2019). On day 1 after oviposition (Fig. 2 A, B), the embryos under hypoxia were smaller and rectangular than those under normoxia (pyriform-shape) (Fig. 2 C). On day 2, appendages were formed in control embryos but not in hypoxia-treated embryos. From day 3, the morphology of hypoxia-treated embryos exhibited almost no changes whereas, control embryos changed dramatically each

day until hatching. This is the reason why hibernating schedule is followed in sericulture, during which oxygen is depleted to low level and remained constant thereafter. During the time of release eggs are gradually kept in higher temperature to regain the activity (Hernández-García et al., 2010). Further, peak ROS levels were observed in diapause eggs within 3 days of oviposition, after which the levels decreased; in contrast, ROS levels in non-diapause eggs were significantly lower. Reactive oxygen species (ROS) are produced by metabolic pathways in almost all cells. As signaling components, ROS are best known for their roles in abiotic and biotic stress-related events. (Mhamdi et al., 2018). Higher concentrations of ROS may cause temporary growth arrest, depression of housekeeping genes and the expression of stress-related genes in cells ROS may induce specific responses and regulate patterns of tissue development during embryonic development (Davies, 1999).



**Fig. 2: Embryo of (A) non-diapause egg, (B) diapause egg under hypoxia and (C) non- diapause egg under normoxia. Numbers 1 to 9 represent days after oviposition.**

1. **Ecdysteroidogenic enzyme and ecdysteroid signaling genes**



**Fig.3: The ecdysteroid biosynthesis pathway and ecdysteroidogenic enzymes in insects**

The biosynthetic pathway of ecdysteroids begins from oxidation of cholesterol into 7‐dehydrocholesterrol (7dC). This initial step is mediated by a *Neverland* (*Nvd*) gene. Between this 7dC and the first upstream compound exhibiting the highly characteristic ecdysteroid structure, diketol, lays the “black box.” This black box includes a series of unidentified and uncharacterized reactions which ultimately result in the oxidation of 7dC to diketol (Ryusuke and Yuko, 2014). Involvement of *CYP307A1* (also called *Spook* (*Spo*) in several insect species)/A2 (also called *Spookier* in *Drosophila*) in the black box, which appears to the rate‐limiting enzyme. Hydroxylation at C25, C22 and C20 is mediated by *CYP306A1* (also called *Phantom* (*Phm*), *CYP302A1* also called *Disembodied* (*Dib*) and *CYP315A1* also called Shadow (Sad), respectively (Nagasawa *et al.,* 1986, Mizuguchi *et al.,* 1987, Sakurai *et al.,* 1989).

The final hydroxylation from ecdysone to 20‐hydroxyecdysone (20E) is catalyzed by a fourth hydroxylase, *CYP314A1*, *Shade* (*Shd)* in peripheral target tissues. Role of different enzymes is discussed here. *Eppase*: Ecdysteroid Phosphate Phosphatase converts ecdysteroid phosphate to free ecdysteroids. The halloween genes: *Spo, Nvd, Phm, Dib, Sad* and *Shd* code for cytochrome P450 enzymes in the ecdysteroidogenic pathway (biosynthesis of ecdysone from cholesterol) (Gu *et al*., 2021, Marchal *et al.,* 2011, Chavez *et al*., 2000). *E75A, E75B, E74A, E74B*: Ecdysone inducible 75A, 75B, 74A, 74B. *Br-C*: Broad- Complex: Encodes family of transcription factors needed for metamorphic processes (Niwa and Niwa, 2014). Receptors: *EcRA* (Ecdysteroid receptor A), *EcRB1* (Ecdysteroid receptor B1) and *USP* (Ultraspiracle). *HR* (Hormone receptor), *Kr-h1* (Kruppel homolog1) and *FTZ‐F1* (Fushi-tarazu factor-F1)

The study clearly demonstrated that the expression patterns of key genes involved in ecdysteroid synthesis (EPPase, Spo, Nvd, Shd) and signaling (EcRA, E75A, E75B, HR3, HR4, FTZ-F1) differ significantly between diapause and HCl-treated (developing) silkworm eggs. In diapause eggs, these genes generally showed either low or transient expression, while in HCl-treated eggs, their expression increased during specific stages of embryonic development, especially the middle to late phases of embryonic development. These patterns were also consistent with non-diapause eggs, confirming that the differences in gene expression are directly linked to the diapause state. Similar observations in other insects, such as *Locusta migratoria* (Petryk *et al*., 2003, Lenaerts *et al*., 2016), where ecdysteroid levels are lower in diapause eggs. The results highlight the critical role of stage-specific regulation of ecdysteroidogenic and nuclear receptor genes in controlling embryonic diapause in silkworms indicating development arrested at the G2 phase of the embryonic cell cycle (Jarvela *et al.*, 2017). To maintain this arrested state, the production and activation of ecdysteroids must be minimal. (Nakagaki *et al*., 1991, Jarvela and Pick, 2017)

1. **Expression analysis and functional identification of diapause genes**

*B. mori* diapause is based on the principle that silkworm offspring diapause is regulated by the environmental conditions experienced by the parents during the embryonic period was constructed Jiang *et al*., 2019. The gene product of *BGIBMGA003835* is UDP-glycosyltransferase (UGT), which catalyzes the transfer of the glucuronic acid group of UDP-glucuronic acid to a small hydrophobic molecule. The gene product of *BGIBMGA012335* is related to the solute carrier family 35 member F6(SLC35F6). It may be involved in transmembrane transport of glucose, amino acids and other substances (Parvy *et al*., 2014). The gene product of *BGIBMGA002426* is mannosyl-oligosaccharide α-1,2- mannosidase, which is involved in the procession of protein or peptide in endoplasmic reticulum. The product of *BGIBMGA012996* is glucose dehydrogenase (FAD, quinone) which is important for glucose metabolism and energy supply (Hahn and Denlinger 2011).

The expression levels of *BGIBMGA003835* and *BGIBMGA012335* were significantly higher in the PDD (progeny diapause destined) group than the PNDD (progeny non-diapause destined) group, with 8-fold and 45-fold differences seen for the two genes, respectively indicating that the manufacture and storage of glycogen is increased in diapause eggs (Lin *et al*., 2009). The expression levels of *BGIBMGA002426* and *BGIBMGA012996* were significantly decreased in the PDD group compared with PNDD group, with 7-fold times and 16-fold differences, respectively indicating that activity of Mannosyl- oligosaccharide α- 1,2- mannosidase and Glucose dehydrogenase activity is reduced in diapausing eggs (Chen *et al.,* 2017).

1. **VECT-mediated artificial breaking of silkworm egg diapause**

Zhang *et al*., (2022) designed a novel artificial corona instrument used to successfully disrupt the diapause of newly laid and refrigerated eggs from various silkworm strains (including Chinese and Japanese lineages). Subsequently, they invented a more eco-friendly, safer, less expensive and handier strategy with broad adaptability, named very early corona (electric field) treatment (VECT) at voltage 12 Kv, pole pitch, 8 mm to prevent eggs from entering diapause by incorporating corona treatment on newly laid silkworm eggs within 4 h of oviposition. The maximum average hatching rates of the larvae reached 95.77%–97.15%, which was comparable to the effect of corona or HCl treatment in preventing the diapause of eggs at 20 h after oviposition**.** VECT-mediated artificial incubation of eggs within 4 h of oviposition revealed that the larval hatching rates of Dazao eggs collected at 0.5–1.5 h after oviposition increased significantly with the variation in the corona treatment times from 5 s to 1min 30s. The average maximum hatching rates of the larvae reached 88.57–93.19 per cent. However, when the length of the corona treatment time was longer than 2min, the larval hatching rates decreased significantly. Moreover, the hatching rates at 2–4 h after oviposition increased significantly with the variation in corona treatment times from 5s to 30s and reached the maximum following treatment for 30s. Subsequently, the larval hatching rates of DZ eggs did not change significantly with an increase in the corona treatment times (from 30s to 5 min).

The mechanism of corona treatment to disrupt the diapause of silkworm eggs is unclear. However, it can be speculated that corona treatment disrupted the serosal layer formation which is key characteristic of diapuase initiation. Some studies have speculated that the electric current may cause a conformational change in the diapause hormone in the embryo, leading to its inactivation, thereby inducing the embryo to disrupt diapause and initiate development (Yang *et al*., 1994). Other studies have speculated that the free radicals generated by corona discharge in silkworm eggs are one of the important factors that disrupt diapause (Ye *et al*., 1996, Hernandez-Garcia *et al*., 2010). It has been reported that the mortality of silkworm eggs after corona treatment is low, which may be due to the disinfection and sterilization effect of ozone in the air (Chen and Zhu, 1997). However further studies regarding build up of effects of radiation due to continuous treatment generation after generation need to be demonstrated. Also, the large-scale implication of this technique and success in the grainage will boost sericulture worldwide.

1. **CONCLUSION**

Diapause acts as a strategy for insects to withstand adverse climate and ensures their long-term survival. Studies in molecular biology have shed light on the gene regulatory network underlying diapause in silkworms. Unraveling the intricacies of gene expression during diapause opens avenues for targeted genetic interventions. Manipulating key genes involved in diapause regulation holds the potential to modulate or control this process, offering novel strategies for improving silk production and enhancing the resilience of silkworms to environmental challenges.

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