

Variation of embryonic diapause induction in bivoltine silkworm *Bombyx mori* L (Lepidoptera: Bombycidae) under controlled conditions

Kiran Rudramuni*, Bharath Kumar Neelaboina, Shivkumar, Mir Nisar Ahmad, and Sukhen Roy Chowdhury

Silkworm Improvement Section, Central Sericultural Research and Training Institute, Central Silk Board, Pampore, Jammu and Kashmir, India

Abstract

Mulberry silkworm is classified into uni, bi and multivoltine based on the frequency of diapause incidence. The variation in the incidence of diapause in bivoltine silkworm provides a unique opportunity to study the process of evolution of adaptive plasticity towards seasonal variations. The diapause expression in bivoltine silkworm is highly variable and is determined by environmental factors experienced by the maternal generation. Diapause in natural populations is functionally associated with the overwintering mechanism that facilitates survival in harsh winter conditions. In contrast, under standard commercial rearing conditions, the domesticated bivoltine silkworm is known to enter diapause in every generation. This paper presents a short review of the literature dealing with the role of temperature, photoperiod, diapause hormone and its receptor in diapause induction. Also, we briefly review the incidence of non-diapause eggs in bivoltine silkworm under controlled conditions.

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Introduction

Voltinism in mulberry silkworm, *Bombyx mori* L (Lepidoptera: Bombycidae) typically exhibits a variety of patterns. It is classified into univoltine (one generation per year), bivoltine (two generations per year) and multivoltine (multiple generations per year) based on variation in the induction of diapause (Morohoshi 1969; Yoshitake 1970). Diapause is considered as one of the most substantial alternative developmental pathways used as a means to survive unfavourable environmental conditions.

In mulberry silkworm, diapause occurs during embryonic development as an overwintering mechanism (Yamashita and Hasegawa 1985; Yamashita and Yaginuma 1991; Xu *et al.* 1995a, b; Shiomi *et al.* 2015). Diapause eggs are characterized by a cessation of embryonic development and non-diapause eggs precede embryonic development without any interruptions (Horike and Sonobe 1999; Sasibhushan *et al.* 2013).

In general, univoltine and bivoltine are suited to cold and temperate regions, and multivoltine are suited to tropical regions. Under natural conditions, the eggs from univoltine and bivoltine

*Corresponding author.

Kiran Rudramuni

Silkworm Improvement Section, Central Sericultural Research and Training Institute, Central Silk Board, Pampore, Jammu and Kashmir, India

Tel: 91-886-1650-760

E-mail: kiranrmv1990@gmail.com

moths in temperate regions hatch during the spring season in concert with the growth of mulberry leaves. The moths emerging after completion of larval and pupal stage lay diapause eggs (Univoltine) and non-diapause eggs (Bivoltine). The moths emerging from non-diapause eggs of bivoltine moths lay diapause eggs in the autumn season. Multivoltine eggs in tropical regions never enter diapause and complete multiple generations annually (Otsuki and Sato 1997; Kamili and Masoodi 2000; Chauhan and Tayal 2017).

Genetic variation plays an essential role in the process of adaptation to environmental heterogeneity. Genetically, a uni, bi and multivoltine are differentiated based on the presence of 3 multiple alleles V1, V2 and V3 involved in the control of the brain esophageal ganglion- diapause hormone system. Additionally, H-S (sex-linked), H-1, H-2, H-3 (autosomal), *Lm* and *Lm^e* are also reported to determine voltinism (Morohoshi 1969; Aswath 2005). From here, this paper will specifically focus on bivoltine silkworms.

Embryonic development of diapause eggs

Development, reproduction and induction of diapause under favourable seasons is an essential requirement for insects of temperate and colder climate regions. The evolution of embryonic diapause as an alternative developmental pathway in mulberry silkworm facilitates survival in freezing temperature during winter. The timing of diapause is strongly influenced by the rate of development, reproductive schedule, host availability, and seasonality. Bivoltine silkworm under temperate conditions is composed of two distinct populations. The difference among the populations is identified based on the incidence of diapause and occurrence of seasonal polyphenism in wing patterns (brown, dark-brown scales on wings) (Tsurumaki *et al.* 1999). The environmental factors experienced by the maternal generation during the egg, larva and pupal stage typically determine the embryonic diapause of next-generation (Otsuki and Sato 1997; Zhao *et al.* 2014; Akitomo *et al.* 2017).

According to previous findings, temperature, humidity, photoperiod, and nutrition are identified as the most reliable cues concerning diapause induction. Low temperature and short day length typically indicate the availability of a favourable environment for next-generation, whereas high temperature and long daylengths cue for a cessation of embryonic development (Kogure 1933; Shimizu 1982, 1991). The neuroendocrine

functions involved in the diapause induction are determined based on the thermal and photic stimulation received by the developmental stages of silkworm (Tauber *et al.* 1986 cited in Shiomi *et al.* 2015). Since the cocoon quality of silkworm that lay diapause eggs are superior to that of silkworm that lay non-diapause eggs, the demand for diapause eggs in the sericulture industry is high (Otsuki and Sato 1997).

In diapause eggs incubated at 25°C, the embryonic diapause occurs at an early embryonic stage i.e. after 3 days of oviposition (Coulon 1967 cited in Dorel and Coulon 1988; Kitazawa *et al.* 1963 cited in Nakagaki *et al.* 1991). The developmental stages in the process include fertilization (0-2 hours), cleavage (2-10 hours), blastulation (10-20 hours), yolk cleavage (20-24 hours), daruma (24-30 hours), and kokeshi (30-40 hours). On the third day, the embryos cease further development and enter the final stage here, identified as the pre-diapause period (Otsuki and Sato 1997). The mitotic activity in embryonic cells in the period between 24 and 72 hours of oviposition gets reduced, and cell cycles are arrested at the G2 phase (Nakagaki *et al.* 1991).

The interaction between Diapause Hormone (DH), a 24 amino acid peptide amide secreted from maternal suboesophageal ganglion, and DH Receptor (DHR1) expressed in ovaries are crucial for the determination of embryonic diapause. DH, encoded by Diapause hormone -Pheromone Biosynthesis Activating Neuropeptide (DH-PBAN) is a sensitive and specific signalling molecule for DHR. Interestingly, DH and DHR1 are found both in diapause and non-diapause silkworms. However, the level of DH and its interactions with DHR vary in developmental stages involved in the determination of embryonic diapause (Hasegawa 1957; Sato *et al.* 1993; Xu *et al.* 1995a; Yamashita 1996; Homma *et al.* 2006; Kitagawa *et al.* 2005; Watanabe *et al.* 2007; Sato *et al.* 2014; Jiang *et al.* 2016; Gong *et al.* 2017).

A considerable discrepancy exists between the level of DH secretion in silkworms from 25°C and 15°C. According to the study by Xu *et al.* (1995a), DH-PBAN mRNA content in silkworm destined to lay diapause eggs (eggs incubated at 25°C) showed peaks during embryo, larvae and pupal-adult development. In contrast, DH-PBAN mRNA content in non-diapause silkworms (eggs incubated at 15°C) showed a peak only during pupal-adult development. The study also reported that interruption of the incubation period temperature from 25°C to 15°C causes a decrease in the DH-PBAN mRNA content in embryo and pupae and the incidence of diapause eggs. On the

contrary, Morita *et al.* (2003) and Kitagawa *et al.* (2005) reported insignificant differences in the levels of DH and DH-PBAN mRNA during embryonic development. However, the difference observed in the DH levels was restricted to the embryonic stage, and considerable levels of difference in DH of subesophageal ganglion during post-embryonic development were also observed by Kitagawa *et al.* (2005) similar to Xu *et al.* (1995a).

Extensive research has been conducted to reveal the difference between diapause and non-diapause eggs. Rather than attempt to summarise these results, we refer the reader to the following references for details. Role of DH, DH-PBAN, DHR1, *Ptix1*, *Bombyx* Dopa decarboxylase (*BmDdc*) in diapause induction (Xu *et al.* 1995a, b; Yamashita 1996; Shimizu *et al.* 1997; Noguchi and Hayakawa 2001; Morita *et al.* 2003; Zhang *et al.* 2004; Kitagawa *et al.* 2005; Shiomi *et al.* 2007, 2015; Yokoyama *et al.* 2021), subesophageal ganglion neuropeptides (Shiomi *et al.* 2015), dopamine and gamma-aminobutyric acid (Hasegawa and Shimizu 1987; Shimizu *et al.* 1997; Noguchi and Hayakawa 2001; Ichikawa 2003; Cui *et al.* 2021), N6-methyladenosine modification (Jiang *et al.* 2019), Ecdysteroid content (Coulon 1984, 1988; Makka and Sonobe 2000), the difference in proteins content (Fan *et al.* 2013), the morphological difference (Takesue *et al.* 1971, 1976), carbohydrate metabolism (Chino 1957, 1958; Yamashita *et al.* 1972; Yaginuma and Yamashita 1978; Sonobe *et al.* 1979; Yaginuma *et al.* 1990a, b; Abraham *et al.* 1992; Su *et al.* 1994; Horie *et al.* 2000; Homma *et al.* 2006).

Factors affecting diapause induction

Diapause induction is most affected by temperature and photoperiod, followed by humidity and nutrition. Among the developmental stages, the bivoltine silkworm embryo is considered as the most sensitive stage for environmental stimuli (Fukuda 1953; Otsuki and Sato 1997; Denlinger *et al.* 2012). Moths emerging from incubation of eggs at a temperature of 15°C under continuous darkness and 25°C under continuous illumination lay non-diapause and diapause eggs (Watanabe 1919, 1924; Kogure 1933; Fukuda 1953). The intermediate temperature of 20°C under continuous illumination and darkness results in diapause and non-diapause eggs (Kogure 1933; Yamashita and Hasegawa 1985; Shiomi *et al.* 2015; Yamashita 1996 cited in Zhao *et al.* 2014; Morita *et al.* 2003; Kitagawa *et al.* 2005). Similarly, moths developed at 23°C under long-day conditions, 16-hour light and 8-hour dark (16L:8D) and short-day

conditions (10L:14D) laid diapause eggs (Tsurumaki *et al.* 1999). According to Watanabe (1924), the incubation temperature of 24-27°C effectively induces diapause. This is because the incubation temperature of 25°C causes DH secretion in subesophageal ganglion resulting in induction of diapause in resulting embryos (Yamashita and Suzuki 1991; Pearse *et al.* 1987 cited in Xu *et al.* 1995b). The molecular mechanism involved in the induction of diapause with respect to temperature was relatively unknown until recently Sato *et al.* (2014) reported the role of *Bombyx* Transient Receptor Potential A1 (*BmTrpA1*) activation in the induction of diapause in progeny. The study reported that the *BmTrpA1* ortholog that acts as a thermosensitive transient receptor potential channel gets activated at a temperature above 21°C. The study also reported that RNAi of *BmTrpA1* affects the release of diapause hormone during the development of pupa and adults. The results of various combinations of temperature and photoperiod regimes can be read from Kogure (1933) (Table: 27-38, Otsuki and Sato (1997) (Tables 4.5, 4.6), Aswath (2005) (Table 2), Singh (2004) (Table: 7.3), Egi *et al.* (2014) (Table: 1, 2, 3).

Non-diapause eggs under controlled conditions

The occurrence of diapause and non-diapause phenotypes in bivoltine silkworm is determined by specific environmental cues. Larval hatching in natural populations of bivoltine silkworm takes place during spring in concert with the growth of mulberry leaves. The moths emerging from this generation lay non-diapause eggs. Interestingly, the occurrence of the non-diapause phenotype is not restricted to natural populations and has also been reported under controlled conditions by several authors. For instance, a few moths from 2020 spring, summer and autumn rearing laid diapause, non-diapause and the mixture of diapause and non-diapause eggs (Fig. 1). These silkworm races from different origins are maintained under germplasm, breeders stock, and various other breeding/ research programmes in Central Sericultural Research and Training Institute, Pampore. The silkworm larva that emerges after hibernation (cold storage hibernation) are reared on mulberry varieties KNG, Ichinose and Gowsherami at a temperature ranging between 25°C and 28°C (70-90% RH) without specific photoperiod control. The mature fifth instar larvae are collected manually and mounted on collapsible plastic mountages for spinning of cocoons. The temperature during the process is maintained at 25°C. The moths emerging from cocoons are allowed to mate and the eggs laid

are eggs incubated at 25°C to maintain a diapause state. This incubation method is often adopted in bivoltine silkworm rearing for obtaining diapause eggs.

A total of 635 disease-free layings (DFLs) were produced from 18 races. Considering the difficulty of counting all the eggs from 635 DFLs, the data in the table is restricted to non-diapause eggs. The number of non-diapause eggs in the present observation from 18 races was 6871. Assuming that all moths laid around 500 eggs, the percentage of non-diapause eggs was estimated to be 2.16 [6871/(500x635) x100].

In season-wise comparison, the number of moths laying non-diapause eggs during spring and autumn was considerably higher than in summer. The total number of moths from spring, summer and autumn were 8501, 1239 and 1331 respectively. The percentage of moths laying non-diapause eggs in spring, summer, autumn was 0.56 [48/8501x100], 0.10 [01/1239x100] and 0.68 [09/1331x100]. It is worth taking note that, Kashmir experiences temperate climatic conditions composed of spring, summer, and autumn seasons with clear distinctions in temperature and sunlight. Spring season under temperate conditions attributes to the short days and low-temperature regimes in comparison to summer. The day length of autumn in comparison to spring is longer, and the temperature is lower in comparison to summer. These observations are in principle with the fact that long days and high-temperature regimes are known to induce diapause in bivoltine silkworm.

On contrary, under tropical conditions, the sporadic incidence of bivoltine silkworm laying non-diapause eggs has been reported by Muniraju and Mundkur (2018). Similar observations have been made in grainages located in tropical regions of South

India (personal communication). Similarly, Tsurumaki *et al.* (1999) reported the occurrence of diapause, non-diapause and mixed eggs of diapause, non-diapause in moths from bivoltine race Daizo developed at the temperature of 25°C and 28°C under long-day conditions (16L:8D). Unlike temperate regions, bivoltine silkworm in tropical conditions are known to enter diapause in every generation (Muniraju and Mundkur 2018). The explanation for the occurrence of non-diapause eggs at high-temperature regimes remains relatively unknown/ unexplained. According to Muniraju and Mundkur (2018) “Extensive studies on diapause mechanism are undertaken in temperate conditions, but under tropical conditions, the studies are scanty.”

Interestingly, the occurrence of non-diapause eggs is not restricted to high temperatures. In an experiment involving several bivoltine races from Japan and China, Kosegawa *et al.* (2000) reported the occurrence of diapause, non-diapause and mixed batches containing both diapause and non-diapause eggs at the low temperature of 15°C with constant darkness. Similarly, considerable differences have been observed even in the transient temperature between 15°C and 25°C. For instance, Tsurumaki *et al.* (1999) reported that moths developed at 23°C under long day (16L:8D) and short-day conditions (10L:14D) laid diapause eggs. On the contrary, in the study by Fukuda and Takeuchi (1967) incubation temperature of 23°C under illumination resulted in the development of both diapause and non-diapause egg producers.

A comparable result has been reported by Jayaswal (1994) and Subramanya (1998) in tropical multivoltine stocks. Jayaswal observed that moths from indigenous multivoltine silkworm races known to lay non-diapause eggs laid a high percentage of diapause eggs during May-July experiencing shorter days



Fig. 1. Representative images of DFLs containing a) diapause b) non-diapause and c) mixture of diapause and non-diapause eggs

and low-temperature regimes. Similarly, in a translocation experiment, moths from tropical multivoltine silkworm races were observed to lay few diapause eggs in the autumn season of Japan. These observations indicate that the multivoltine silkworms of tropical origin also possess overwintering mechanisms similar to univoltine and bivoltine silkworms. Murakami (1987) and Murakami and Ohtsuki (1989) reported that voltinism is inherited maternally, where photoperiod is a primary factor, and other physical variables act as a secondary factor for the determination of voltinism.

Conclusion

In conclusion, embryonic diapause in bivoltine silkworm is a unique process of seasonal polyphenism that is influenced by several factors. Under natural conditions, bivoltine silkworm originating from temperate regions completes two generations annually. The female moths from the first generation developed at low temperature under short days lay non-diapause eggs and the second generation developed at high temperature under long days lay diapause eggs to facilitate survival in the resource-depleted environment. Under controlled conditions, the exposure of embryonic and post-embryonic developmental stages to the temperature of 25°C and above effectively induce diapause in the progeny. The occurrence of non-diapause and mixture of diapause and non-diapause eggs under controlled conditions could be due to the exposure of embryonic or post-embryonic developmental stages to transient temperature and photoperiod. Considering the specific effects of temperature and photoperiod regime and its association with the stimulation of DH secretion in embryonic and post-embryonic development, it is safe to conclude that a bivoltine silkworm is most sensitive for environmental factors that influence diapause induction.

Conflict of interest

The authors have no conflicts of interest.

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