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[Review Article]

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Occurrence of Unfertilized Eggs in the Mulberry Silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae)

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Quality of seed in the mulberry silkworm, *Bombyx mori* (L.) is determined by many important factors, wherein unfertilized eggs play an important role. Unfertilization of eggs are caused by several reasons such as, abnormality in the sexual organs of the male and female, abnormal development of the micropylar end of the egg , unfavorable environmental conditions during spinning, cocoon preservation, imperfect handling of moths, mating, ovipostion, cold storing of pupae / moths and indiscriminate use of male moths etc. Though the presence of unfertilized eggs would in no way affect the fertilized ones and their quality directly, the frequency of their occurrence underrates the quality and brings down the hatching percentage. Lower the occurrence of unfertilized eggs, higher is the rating of seed quality. Of the various intrinsic and extrinsic factors and events involved in egg deposition of an adult silk moth, mating is an instinct and a biological obligation for the ultimate perpetuation of the species and a must to provide stimulus for oogenesis and bring about biochemical changes in the spermatophore of the silkworm in order to ensure the presence of sufficient number of normal sperms and testicular fluid in the female reproductive organ, activating ovulation and accelerating oviposition behavior and egg deposition. An attempt has been made in this article to briefly elucidate the characteristics of unfertilized eggs, causes of their occurrence and its impact as well as the significance in silkworm seed production.

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Introduction

Silk, one of the bio-resources for human life has always occupied a place of pride and there have been incessant endeavors to improve silk both its quality and quantity. Quality silkworm seed is the basic input to harvest a successful cocoon crop. Quality refers not only to the disease free nature of the layings but also to the occurrence of more viable eggs, uniform hatching, robust larvae, quality cocoons and stable cocoon crops (Singh and Saratchandra, 2004). Quality of seed is determined by many factors of significant importance wherein unfertilized eggs play an important role.

The mated female moth of the mulberry silkworm (*Bombyx mori*) lays mostly fertilized eggs (which produce viable progeny) and a very small quantity of unfertilized and dead eggs. In diapausing breeds, the fertilized eggs are pigmented and can easily be differentiated after about 48 hrs of oviposition from unfertilized eggs. Consequent to a function of fertilization, after the appearance of amnion and serosal layers, the specific pigment 'ommochrome' spreads in the serosal cuticle, which is expressed as deep / dark brown in color (Singh *et al.*, 2002). Devoid of fertilization, apparently in the unfertilized eggs, the egg cuticle remains as such and eggs oblige to retain the same colour imparted at the time of oviposition. On the contrary, in non-diapausing, the eggs are non-pigmented due to absence of ommochrome pigment and thus the discrimination of fertilized and unfertilized eggs is rather difficult. It can be distinguished only when the eggs reach the pin-head stage in the course of development.

The unfertilized eggs remain in their original colour while the fertilized ones acquire the blue color as a result of sclerotization of the cuticular layers of fully developed embryos. In general, under optimum conditions of management, the percentage of dead and unfertilized eggs is very low in a given population. However, it could attain significantly higher proportions when the insect is exposed to unfavorable environmental conditions for a good number of other reasons.

Occurrence of unfertilized eggs or the phenomenon of sterility is however natural among any living system. Though the presence of unfertilized eggs would in no way affect the fertilized ones, the frequency of their occurrence underrates the quality. Fertilized egg is a combined product of the egg cell from the female parent and the spermatozoan of the male parent. It is well known that mating is indispensable for obtaining fertilized eggs. When a normal male and female moth copulate, the male perform an ejaculation in an hour's time by discharging the seminal fluid containing two types of spermatozoa (eupyrene sperm bundles and loose apyrene sperm) accompanied by essential heterogeneous viscous secretions which are derived from the various glands of the male reproductive system consisting of glycogen, arginase, endopeptidase, exopeptidase etc. These secretions promote well-timed metabolic events for sperm maturation, motility of apyrene sperm and dissociation of eupyrene sperm bundles through lashing / flagellating movements of apyrene sperm inside the bursa-copulatrix. (Singh *et al.*, 2002). After dissociation of the eupyrene sperm bundles and consequent maturation inside the bursa-copulatrix, the sperm escape out of the spermatophore as a mass to reach ductus seminalis. Owing to the peristaltic movements exhibited by ductus seminalis, the mass of spermatozoa escapes across vestibulum and begins to pour into the spermatheca (in one hr after copulation ended). After that it gradually increases to fill the spermatheca in about 2.5 hrs after end of the copulation (Suzuki *et al.*, 1996). The spermathecal duct also involves peristaltic movements, assist sperm migration towards spermatheca (Osanai *et al.*, 1990) and these movements occur at a constant frequency after copulation (Suzuki *et al.*, 1996).

Omura (1938) reported two pathways in the spermathecal duct i.e. one where the sperm move from vestibulum to the spermatheca and the other from receptaculum seminis to the vestibulum, the eggs descend down from the common oviduct during the process of oviposition. As the eggs descend down from the ovarian tubules via lateral oviducts into the common oviduct and then in to the vestibulum, the spermatheca contracts releasing large number of sperms on the egg near the micropylar aperture (Narashimhanna, 1988). The sperm penetrates the ovum through the micropylar opening to accomplish further proceedings culminating into fertilization. Polyspermy is usual in mulberry silkworm. However, when there are more than two spermatozoa in one egg, only one of them participates in the fertilizing event and the superfluous ones will degenerate. Seldom, two or more sperm nuclei join resulting in the production of polyploids or dispermic androgenic individuals (Sakaguchi, 1978).

It is apparent that in some eggs, there are no nuclei, which are destined; to become unfertilized eggs i.e. eggs either did not receive the sperm or the sperm disintegrate inside the egg. It may be just a coincidence that when the eggs are descending down through the common oviduct into vestibulum, a few of them, unlucky, fail to receive the sperm, as the eggs stay there for a brief period of 2-3 seconds (the delay is the time required for the mother moth to verify the substratum in all the three dimensions through its sensory hairs present on the anal papillae in order to forward a signal about the availability of clear space for the release of eggs) as they are pushed down by descending eggs and hence they become unfertilized eggs. Thus, any egg contained in the ovarian tubules could transcend down to become an unfertilized egg.

The sperm, which has penetrated into the egg, remains lodged in the anterior part of the egg until the completion of the egg pro-nucleus. In the mean time, the tail of the sperm separates from the head, near the top of the egg a centrosome appears and an aster is formed. The sperm head gradually swells into a spherical body, forming the male pro-nucleus. As soon as the maturation division is completed, the male and female pro-nuclei approach and finally fuse accomplishing the vital function of fertilization. They unite to form the zygote with twice as many chromosomes and thus creating a fertilized egg.

In this article an attempt has been made to briefly review the relevant information available on the occurrence of unfertilized eggs in *Bombyx mori* to provide annotated outline of various causes involved.

Parthenogenesis

Parthenogenesis (virgin origin) is derived from the Greek word 'parthenos' means virgin. In many animals the egg develops into an individual without fertilization (Chowdhury, 1989). Such development of the ovum without fertilization is called parthenogenesis (Singh *et al.*, 1994, 1997) and the individuals that are produced by this method are called 'parthenotes' (Retnakaran and Percy, 1985; Gangopadhyay *et al.*, 2005). Parthenogenesis is a rare phenomenon in animals and is a normal means of reproduction in some lizards (Darevskii and Kulikova, 1961) and several insects (Chapman, 1982). Parthenogenesis has been reviewed by many workers (Strunnikov, 1975; Cuellar, 1977; Clement, 1982; Chowdhury, 1989). In silkworm, the parthenogenesis is divided into three types (Strunnikov, 1983) viz., ameiotic parthenogenesis without reduction in the number of chromosomes (usually in thermally activated ova); meiotic parthenogenesis having maturation divisions (activated by low temperature) and gynogenesis requiring stimulation by spermatozoa for activation of ova.

Suomalainen (1950) classified parthenogenesis based on reproduction (occasional or tychoparthenogenesis, obligatory parthenogenesis, facultative parthenogenesis, rudimentary or incomplete parthenogenesis and artificial parthenogenesis); sex determination (arrehenotoky, thelytoky and amphitoky or deuterotoky) and cytology (generative or haploid parthenogenesis, somatic or diploid parthenogenesis, apomictic or ameiotic parthenogenesis and automictic or meiotic parthenogenesis).

When the female moths of silkworms are kept isolated after emergence and are not mated with the males, they begin to lay eggs after lapse of some time. The rate of oviposition is significantly slower than that of the normal mated females, yet they manage to lay most of the eggs in a few days of week's time. These eggs are indeed unfertilized eggs and fail to transform into embryo and do not hatch. However, in rare cases, embryos are formed in the eggs without following the normal path of development and young larvae hatches out (Aruga, 1994).

Alternatively, if unfertilized eggs are stimulated by immersing in HCl or in hot water, the percentage of the embryo formation and hatching of larvae increases. This phenomenon is referred to as 'parthenogenesis'. These silkworms obviously begin their development from the egg nucleus only and form the embryos. Parthenogenesis in silkworm was experimented by Kawaguchi (1934). The procedure involved heat activation of unfertilized eggs extirpated from the ovarian tubules and immersion into hot water for 18 minutes at 46^{o}C. Astaurov (1967, 1969) opined that the treated eggs could undergo parthenogenetic development to the extent of 82% and some even attained sexual maturity. All the parthenogenetic individuals were females of maternal phenotype. However, Kawaguchi (1934) obtained both sexes through parthenogenesis. Merogony also occurs under artificial stimulation where egg nucleus does not participate in the development of the embryo, which instead develops with the egg cytoplasm and sperm nucleus. After mating of the moths, if eggs laid are immediately subjected to high temperature (40^{o}C) stimulation for one hour, merogony occurs (Aruga, 1994).

Unfertilized eggs and causes of occurrence

In the egg, which has just been laid, there is only yolk filling it, but the serosa / amnion nor the chromosomes can be seen. It is only subsequent to fertilization that the cleavage is initiated signaling morphogenesis followed by the formation of blastoderm, germ band and the appearance of the embryonic layer, namely the amnion and serosa. However, in the unfertilized egg, either the sperm is missing or egg nucleus / sperm are not adequately efficient to participate. Thus, none of these developments can be initiated and no morphogenesis proceeds and eventually the unfertilized egg collapse to death in course of time and hence obviously no viable progeny emerge out.

According to Toyama (1909) (as quoted by Tanaka, 1964), the unfertilized eggs are characterized by –

a. No change in egg nucleus and yolk or

b. changes in color a little but nuclei do not undergo any division and yolk separates to irregular yolk balls or

c. egg nucleus divide and produce many yolk nuclei but do not form germ band or even sometimes form imperfect germ band but do not develop further.

Kovalev (1970) stated that unfertilized eggs are the result of some abnormality in the sexual organ of the male and female or abnormal development of micropylar end of the egg. The unfertilized eggs arise besides due to unfavorable environment during the period of spinning, cocoon preservation, imperfect handling of moths during mating and oviposition, cold storing of pupae / moths and indiscriminate use of male moths etc. It is also reported that the unfertilized eggs are found in large numbers among the eggs laid at the end of oviposition as compared to the eggs laid at the beginning of oviposition by the same female moth (Ming, 1994). The various possibilities on the occurrence of unfertilized eggs are discussed briefly as under.

Temperature is one of the most important abiotic factors with pervasive effect and plays a vital role in the physiology of silkworms. More so, the silkworm represents poikilothermic species, most of whose physiological processes are governed by temperature. Wigglesworth (1972) stated that temperature range between which reproduction occurs are often much narrower than that in which the other activities of the same species remain normal. Reproduction as well as development of an insect is temperature dependent and hence, it immensely influences the dynamics of insect population. Thus, the various operations pertaining to seed production are performed within optimum limits of temperature to avoid reduction in egg yield and fertility rate (Kamble, 1997).

Both low and high temperature during silkworm rearing, cocoon preservation or egg production causes unfertilized eggs (Yokoyama, 1963). The impact of higher temperature is more intense and prolific than the lower one. Number of investigations points to the fact that exposure of male silkworms at higher temperature from the time of spinning to pre-pupal period (32-33°C) brings male sterility (Sugai and Kiguchi, 1967; Sugai and Hanaoka, 1972; Sugai and Takahashi, 1981; Das *et al.*, 1996) and results in increase in the occurrence of unfertilized eggs (Jolly, 1983; Narashimhanna, 1988; Biram Saheb and Gowda, 1987; Ming, 1994). The pre-pupal stage i.e. spinning to pupation is the most sensitive stage of the male silkworm to have sterilizing effect at high temperature followed by the pupal period. It is interesting to observe that high temperature does not inflict the sterilizing effect in the larval period from hatching to the middle of the fifth instar (Sugai and Hanaoka, 1972). Even the seed cocoons when exposed to high temperature of 35°C or in combination with low relative humidity, causes decline in the egg recovery besides corresponding increase in the incidence of unfertilized eggs (Ayuzawa *et al.*, 1972) with more than 90% of the eggs laid at 35°C remain unfertilized. Lowering the relative humidity further increases the percentage of unfertilized eggs at higher temperature (Gowda, 1988). He further stated that when freshly formed silkworm pupae were exposed to 35°C till oviposition, only 31-42% of moths laid normal eggs implying a serious damage to the physiology of pupae.

Higher temperature at the time of egg layings also significantly promotes the occurrence of unfertilized eggs (Kovalev, 1970). Similarly, lower temperature too enhances the frequency of unfertilized eggs (Ayuzawa *et al.*, 1972). The elevation of temperature tends to enhance the rate of development and interferes in various genetic and physiological functions, so either the regular developmental pathways deviate so much that there is no emergence at all or when there is emergence, the moths of either sexes get partially or completely sterilized with the net result of production of high degree of unfertilized eggs (Gowda, 1988).

The copulatory organs of the males treated with high temperature at pre-pupal stage did not exhibit any visible abnormality (Sugai and Hanaoka, 1972). Sugai and Takahashi (1981) after morphological studies of the male copulatory organs reported marked reduction in the production of apyrene sperms with a little change in the quantum of eupyrene sperms. Eupyrene sperms are formed at the end of the larval period till the beginning of the pupal period while the apyrenes are produced only in the pupal period but still high temperature in the pharate pupal stage affected the early stage of formation of apyrenes (Sugai and Takahashi, 1981). High temperature treatment causes marked abnormalities in apyrenes and many of them remain in the testicular chamber without penetrating the basement membrane. Silk moths normally produce about twice as many apyrenes as eupyrenes and the ratio of eupyrene and apyrene is 1:1.84 at 25°C while at higher temperature (32°C), it is 1:0.85 showing that the production of apyrenes was markedly reduced at 32°C during pharate-pupal stage (Osanai *et al.*, 1989). Moreover, they further stated that in the moths treated at 32°C, the number of eupyrene spermatozoa was only 67.2% and apyrenes spermatozoa was 31.2% of that at 25°C moths. Thus, the production of both types of spermatozoa and especially apyrenes are very much lower in the moths treated at 32°C than in those of 25°C-exposed moths (Osanai *et al.*, 1989). Hence, the occurrence of unfertilized eggs is comparatively higher during the hot summer months compared to that in other seasons.

The apyrene play an exceedingly important and a beneficial role. Osanai *et al.* (1987) observed that the apyrenes promote dissociation of the eupyrenes bundles within the spermatophore through their vigorously flagellating movements possibly through the digestion of the prostatic secretion or an endopeptidase. Thus, though a large number of eupyrenes are ejaculated into the bursa-copulatrix of the female moth copulated with the sterile male moth, as the apyrenes quantity is low, the dissociation of eupyrene bundles is not efficient and hence neither eupyrenes nor apyrenes are detectable in the spermatheca. However, when male sterility is partial, significant quantity of eupyrenes and apyrenes prevail and that they also move from the ductus seminalis to vestibulum and to spermatheca and compete for entering the eggs for participating in fertilization. This reveals that the occurrence of unfertilized eggs may be due to either complete breakdown of spermatogenesis, inadequate production or failure to transport the sperms effectively by the male into the genital tract of the female and incapability of sperm movement from bursa-copulatrix to spermatheca or partial movement of sperm to sperm store house. Thus, a reduction in the production of sperm seems to be the main causative reason for male sterility rather than complete cessation in the production of sperms.

When the female pupae or moths were exposed to high temperature of 35°C and crossed with normal males, the egg yield was remarkably reduced. The majority of eggs laid comprised of unfertilized eggs signifying that even in the females, the reproductive function is greatly disturbed and the reproductive organs may fail to transport the sperm to spermatheca. It is also possible that the egg nucleus lost its identity and become unfit for fertilization or disintegrated. High temperature interferes with the developmental process and cause physiological breakdown leading to partial or complete loss of reproductive potential in either sex (Gowda, 1988).

The effect of low temperature on insects is manifold. In case, the temperature during seed cocoon preservation is lower than 20°C, unfertilized eggs will increase and further consolidate into higher proportion at 15°C (Kovalev, 1970; Tazima, 1978; Ming, 1994). For the purpose of synchronized emergence, seed cocoons are cold stored for 3 - 7 days only. Cold storing of seed cocoons besides lowering the percentage of moth emergence, egg recovery and fertility would also enhance the percentage of unfertilized eggs (Ming, 1994; Christiana *et al.*, 2003) even to the extent of 20%.

Chilling of female moths is yet another cause for the occurrence of unfertilized eggs. Chilling at 5°C should be limited to only 2 - 3 days as unfertilized eggs are produced only if it prolongs further (Tazima, 1978). Also observed that male moth can withstand refrigeration for 2 weeks but too long refrigeration causes unfertilized eggs. Contrary to this, Ming (1994) observed that cold storing of male moths beyond 4 - 5 days affected the mating capability besides enhancing the frequency of unfertilized eggs. Gowda (1988) reported that the incidence of unfertilized eggs increases with the increase of the duration of cold storing of male moths.

When the mated female moths are cold stored beyond 4 days, the moths lay unfertilized eggs that increase with the increases in the duration of the cold storage. The increase in the number of unfertilized eggs might be due to the inactivation of sperms present in the spermatheca of the cold stored female moth (Raju *et al.*, 1990). It is also possible that the process of ovulation is so much accelerated that most of the eggs briskly or swiftly escapes out of the vestibulum and hence become unfertilized. Failure to transport the sperms effectively inside the female genital tract and the entire consignment or portion of sperm from the spermatophore may be retained inside the bursa-copulatrix and may not reach the spermatheca due to some defect in the bursa or ductus seminalis or vestibulum. The organs associated with the passage of sperm i.e. ductus seminalis; vestibulum and recepticulum seminis are severely affected and may not fully encourage the transport pathway.

Mating is another important event that if handled inadequately, leads to significant increase in the unfertilized eggs. Mating should be conducted at optimum temperature for better recovery and higher or lower temperature range will produce fewer eggs with higher percentage of unfertilized eggs (Ayuzawa *et al.*, 1972). Singh *et al.* (2003) reported that mating at higher (35°C) or lower (20°C) temperature in combination with optimum relative humidity do not influence the fertility status of male or female and the recovery of eggs and the increase in the number of unfertilized eggs. However, when oviposition and or pairing of moths are conducted above 35°C under optimum humidity conditions, fecundity and fertility were greatly reduced and the number of eggs retained inside the body of the moth increased proportionately.

The success of mating depends on the overall physiological / reproductive / health status of the participating male and female moths, their age, time and duration of mating and the number of times the male moth are used for mating (Singh and Tripathi, 1995; Singh and Saratchandra, 2004). Sufficient time should be allowed for copulation and if it is too short, egg laying will be late and slow and fewer eggs will be laid with many of them being unfertilized (Ming, 1994). Jolly *et al.* (1964) and Singh and Tripathi (1995) observed that lower mating durations (15 - 30 min) caused sterility and opined that 3 - 4 hrs of copulation time should be allowed so that at least two ejaculations from the males are exploited. Mere physical union of male and female moths would not suffice unless there is ejaculation of sufficient quantity of spermatozoa and other heterogeneous viscous secretions into the female bursa-copulatrix including the Oviposition Stimulating Substance (OSS) (Biram Saheb *et al.*, 2005).

Indiscriminate use of male moths is yet another cause of occurrence of unfertilized eggs. Continuous mating without rest between two mating affects fecundity and increases the unfertilized and dead eggs (Subramanyam and Narasimhamurthy, 1987; Biram Saheb and Gowda, 1987; Sreenivasababu, 1993). It is well known that when a sterilized male moth is crossed to a normal female, the eggs recovered are unfertilized eggs (Fugo and Arisawa, 1992). Also, there is increase in the incidence of unfertilized eggs when the female moth is affected with pebrine disease (Jolly, 1983).

Strategies to avoid unfertilized eggs

a) Maintain optimum temperature (25±2°C) and humidity (80±5%) during seed cocoon spinning, preservation and oviposition.
b) Proper photoperiod during pre-eclosion and eclosion period is to be provided.
c) Ensure proper and perfect handling of moths during eclosion, mating and oviposition.
d) Conduct silkworm rearing by adopting recommended package of practices of seed crop rearing.
e) Duration and frequency of mating, age of moth on mating, time of mating, re-use of male moth for successive mating, multiple mating etc should be judiciously decided to harvest quality seed.
f) Indiscriminate use of male moth should be avoided.

g) Surface texture of the substratum should be somewhat smooth for egg laying to avoid delay for the release of descending eggs.
h) Cold storing of seed cocoons, moths and eggs should be avoided. If at all necessary for synchronization, it should be for a minimum recommended period only.

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