International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 14-09-2025, Accepted: 14-10-2025, Published: 29-10-2025
Volume 10, Issue 10, 2025, Page No. 105-120

Molecular regulation of diapause in Insects: Integration of signaling pathways, genes, enzymes, hormones, and epigenetic mechanisms

Dr. Saurav Shome
Assistant Professor, Department of Zoology, Bhairab Ganguly College, Belgharia, Kolkata, West Bengal, India

Abstract
Insect diapause is a dynamic, genetically programmed state of developmental arrest that enables survival under unfavorable environmental conditions. This review provides a comprehensive synthesis of the molecular and physiological mechanisms governing diapause across diverse insect taxa. It examines how metabolic suppression and biochemical adaptations support energy conservation and stress resilience during diapause, including the accumulation and strategic utilization of storage proteins and nutrient reserves. Central to diapause regulations are circadian rhythms and photoperiodic cues, which orchestrate its initiation and maintenance through complex interactions between clock genes and neuroendocrine pathways. Recent advances in understanding the genetic and hormonal regulation of larval diapause as well as the mechanisms of cell cycle arrest during insect diapause are also addressed in this review, highlighting how growth and proliferation are suppressed at the molecular level. In addition, the protective roles of heat shock proteins and antioxidant systems in enhancing stress tolerance during diapause are explored. A critical section of this review focuses on the roles of key hormonal signals—including prothoracicotropic hormone (PTTH), ecdysteroids, juvenile hormone (JH), and their upstream regulators in the corpora allata—in modulating diapause entry, maintenance, and termination. The transcription factor FoxO is identified as a central integrator of stress signaling, nutrient sensing, and developmental arrest. The regulatory functions of lesser studied but increasingly relevant signaling pathways, such as adipokinetic hormone (AKH), corazonin, and Wnt signaling, are also discussed. Finally, the review explores how epigenetic modifications, chromatin remodeling, and non-coding RNAs—particularly microRNAs—contribute to the long-term reprogramming of gene expression during diapause. By integrating findings across molecular, hormonal, and physiological domains, this review offers a holistic perspective on the regulatory networks that orchestrate insect diapause, with implications for developmental biology, pest management, and climate resilience in insects.

Keywords: Insect diapause, circadian clock genes, neuroendocrine regulation, FoxO signaling, PTTH, Juvenile Hormone (JH), Ecdysteroids, Heat Shock Proteins, epigenetic regulation

Introduction

Diapause is a genetically programmed developmental arrest that allows insects and other invertebrates to survive unfavorable environmental conditions such as extreme temperatures, drought, or food scarcity. Unlike quiescence, which is a direct response to immediate stress, diapause is an anticipatory and hormonally controlled process, often tightly synchronized with seasonal cues like photoperiod and temperature [1-6]. Diapause involves profound changes in physiology, metabolism, gene expression, and behavior that prepare insects for extended periods of dormancy. Diapause begins and ends under very specific internal and environmental conditions, and can be triggered even when the environment still appears favorable. Diapause usually persists well beyond the return of favorable conditions, allowing insects to conserve both energy and stored nutrients [1-6].

As an evolutionarily conserved survival strategy, diapause helps insects prepare in advance for upcoming adverse seasons. These preparations may include accumulating fat and glycogen, depositing waxy lipids on the cuticle to reduce water loss, suppressing reproduction, lowering metabolic activity, and enhancing tolerance to cold, low oxygen, and other stress factors [1, 3-8]. Some insects may also change color to blend into dry or wintry landscapes, migrate short distances to more sheltered spots, or find protective hiding places to wait out unfavorable seasons. Insects can enter diapause at various life stages—embryonic, larval, pupal, or adult—depending on the species and its environmental adaptations [3-8].

Moreover, diapause is not a single static state, but a dynamic process involving entry, maintenance, and termination phases, each governed by distinct molecular mechanisms. This temporal complexity is reflected in shifts in gene expression profiles, chromatin modifications, and metabolic reprogramming, which vary across tissues and species [1, 3-8].

Recent advances in molecular biology, genomics, and transcriptomics have begun to unravel the complex regulatory networks underlying diapause in insects. Central to this regulation are signaling pathways involving hormones such as juvenile hormone (JH), ecdysteroids, insulin-like peptides (ILPs), diapause hormone (DH), and neuropeptides, which modulate gene expression through transcription factors and epigenetic mechanisms. These signaling pathways interact with environmental inputs via circadian and photoperiodic clocks, allowing insects to make developmental decisions that are both environmentally informed and genetically programmed [1, 5-7, 9, 10].

In a previously published review article, the present author discussed diapause, quiescence, and other hypothermic states, along with the cues that trigger diapause in insects, the types and sequential phases of diapause, and its ecological, physiological, behavioral, and developmental aspects in detail [11]. The present review aims to synthesize

current knowledge on the molecular regulation of diapause across insect species and to highlight both conserved and divergent mechanisms, as well as key hormonal and signaling pathways. By integrating recent findings from molecular biology, endocrinology, and physiology, the author aims to provide a comprehensive understanding of how insect diapause is initiated, maintained, and terminated at the molecular level, and how these regulatory systems have evolved to support developmental plasticity and ecological resilience.

Metabolic Suppression and Biochemical Adaptation in Diapausing Insects

Diapause in insects is marked by a significant drop in metabolic activity, especially in immobile life stages such as pupae and embryos. These stages show the lowest oxygen consumption during diapause — often just 10–20% of the already minimal rate found in non-diapausing individuals. In contrast, the metabolic reduction in diapausing adult insects and larvae is usually less dramatic, typically a 40–50% decrease [8]. In some cases, such as in the overwintering monarch butterfly (*Danaus plexippus*), the metabolic reduction may be as little as 15% [12]. In adult linden bugs (*Pyrrhocoris apterus*), the metabolic rate during diapause is about 25% of the pre-diapause level. Interestingly, in this species, oxygen consumption varies between day and night, with higher rates during the light period (photophase) than during the dark period (scotophase) [13].

The reduced metabolic rate during diapause allows insects to conserve energy and survive for extended periods — sometimes months — without feeding. Diapausing insects are far less active than non-diapausing individuals, and since their development is halted, their energy requirements are significantly lower. For insects that enter diapause during winter, the cold temperatures further reduce energy demands [1, 11].

A key indicator of cellular metabolism is the activity of cytochrome c oxidase — an enzyme involved in the final step of the mitochondrial electron transport chain — which is generally low during diapause. This has been observed across many species — in embryos of the ground cricket (*Allonemobius socius*), larvae of the bamboo borer (*Omphisa fuscidentalis*), pupae of the sweet potato hornworm (*Agrius convolvuli*) and the cotton bollworm (*Helicoverpa armigera*), and adults of *P. apterus* [14-16]. In all cases, either the enzyme's activity is low or the expression of genes encoding cytochrome c oxidase subunits — such as *cox1* — is reduced during diapause. These levels typically rise again once diapause ends, reflecting the reversal of the metabolic suppression characteristic of this state [8, 14-17].

In addition to cytochrome c oxidase, other mitochondrial enzymes also show reduced activity during diapause in insects. Enzymes such as citrate synthase, NAD-isocitrate dehydrogenase, and glutamate dehydrogenase may be suppressed by up to 50%, as observed in the goldenrod gall fly (*Eurosta solidaginis*) [17].

Large-scale gene expression studies have consistently confirmed that ATP-generation genes are commonly downregulated during diapause in insects [18]. A striking example of energy conservation involves the degeneration of flight muscles in certain adult insects during diapause. For instance, in the Colorado potato beetle (*Leptinotarsa decemlineata*), flight muscles in non-diapausing adults grow rapidly and reach full size within eight days. In contrast, in diapausing beetles, these muscles grow only slightly and begin to degrade by day four. The muscle fibers become thin and contain very few mitochondria [19]. Similarly, in the handsome fungus beetle (*Stenotarsus rotundus*), flight muscles fully develop before diapause, enabling the beetles to reach aggregation sites, but then degenerate shortly after arrival [20].

A common feature of diapause in many insect species is a significant increase in expression of the gene encoding phosphoenolpyruvate carboxykinase. This enzyme (PEPCK) functions in anaerobic metabolism by converting oxaloacetate into CO_2 and phosphoenolpyruvate (PEP), a molecule essential for gluconeogenesis. For instance, in *Sarcophaga crassipalpis*, levels of the PEPCK enzyme increase ninefold during pupal diapause. Similar elevations in the *pepck* transcript or its protein product, PEPCK, have been observed in several insect species during diapause. Examples include embryonic diapause in *Aedes albopictus*, ovarian diapause in *Bombyx mori*, larval diapause in *Sitodiplosis mosellana*, and diapause in *Wyeomyia smithii*, *Dendroctonus ponderosae*, *Rhagoletis pomonella*, and *Delia antiqua* [21, 22]. Additionally, elevated PEPCK activity is observed during adult diapause in *Bombus terrestris* and *Drosophila melanogaster* [23].

Metabolomic studies have revealed a broader metabolic shift in insects undergoing diapause. As aerobic metabolism declines, anaerobic pathways become more dominant during diapause. Instead of relying on the oxygen-dependent citric acid (TCA) cycle, diapausing insects switch to pathways such as glycolysis and gluconeogenesis, which are better suited for anaerobic conditions [7-8, 10]. To ensure ATP production and maintain redox balance, diapausing insects increase the activity of pathways like glycolysis, gluconeogenesis, the pentose phosphate pathway, and the PEPCK–succinate route. Diapausing insects primarily depend on stored energy-rich molecules (such as lipids) and rely heavily on β-oxidation of fatty acids, the glyoxylate cycle, and gluconeogenesis to produce essential compounds [7-8, 10].

In the flesh fly (*Sarcophaga crassipalpis*), this metabolic shift during pupal diapause is evident from elevated levels of glucose, alanine, and pyruvate, while levels of fumarate and citrate—key intermediates of the TCA cycle—are significantly reduced. In this species, glucose levels increase nearly 30-fold during diapause compared to non-diapausing pupae. This excess glucose serves as the raw material for producing cryoprotectants such as glycerol and sorbitol, which help the insect survive freezing conditions [24]. Glucose levels similarly rise in other diapausing insect species, including the Colorado potato beetle, the tropical beetle *Stenotarsus rotundus*, and fruit flies (*Drosophila melanogaster*) [10, 23].

Another major metabolic adaptation during diapause involves lipid regulation. Conserving fat and limiting its utilization is critical for surviving prolonged cold periods. At the onset and during the early stages of diapause, fat storage pathways are upregulated in insects, while fat breakdown via β-oxidation is often suppressed—likely as a strategy to preserve energy reserves [10, 11, 13].

A dramatic dietary and metabolic shift can be observed in diapausing insects such as *Culex pipiens*. While non-diapausing adult females are aggressive blood-feeders, diapausing individuals feed only on nectar. This shift is mirrored at the genetic level: the expression of genes

encoding proteolytic digestive enzymes such as trypsin and chymotrypsin is downregulated in diapausing mosquitoes, while the gene encoding fatty acid synthase, which is involved in fat accumulation, is upregulated [25].

Protein synthesis also declines markedly in insects undergoing diapause. Experiments using pulse labeling reveal that both whole-body and brain tissues of diapausing *Sarcophaga crassipalpis* pupae synthesize proteins at much lower rates than actively developing individuals. Similarly, in *Helicoverpa armigera*, genes that repress transcription are activated, while genes that promote translation are suppressed at the onset of diapause. A comparable trend is seen in adult *Neocalanus flemingeri*, where reduced expression of proteins such as serine protease and E3 ubiquitin ligase suggests slower protein turnover [8, 26]. Meanwhile, in the brine shrimp *Artemia franciscana* and the copepod *Neocalanus flemingeri*, ubiquitin-dependent protein degradation is suspended during diapause [26].

Storage Proteins and Nutrient Conservation During Diapause in Insects

As diapause begins, many insects produce large quantities of storage proteins that accumulate in their hemolymph. These "diapause-associated proteins" are significantly more abundant in diapausing individuals than in their non-diapausing counterparts, as observed in the boll weevil *Anthonomus grandis* [5, 7, 10, 11, 27]. First described in *Diatraea grandiosella* larvae, these proteins are now known to be present in various species, though they are not universally present. They are primarily synthesized in the fat body and released into the hemolymph, where they remain throughout diapause. In non-diapausing insects, the same proteins are produced but quickly used for development; in diapausing individuals, these proteins persist until diapause is over and development resumes [5, 7, 10, 11, 27].

Among these 'diapause-associated proteins,' the most notable are the hexamerins, composed of six identical or similar subunits, each weighing 70–80 kDa. Hexamerins are categorized into arylphorins and methionine-rich storage proteins. Arylphorins, rich in aromatic amino acids (18–25%), are predominantly found in diapausing adults of *A. grandis.* On the other hand, methionine-rich hexamerins contain 4–11% methionine in Lepidoptera and are more common in *Chilo suppressalis* larvae [5, 7, 10, 11, 27].

During diapause, these specialized storage proteins serve as reservoirs of nitrogen and amino acids—crucial resources for building new tissues during metamorphosis or for supporting reproduction once diapause ends [27].

Because insects cease feeding or feed very little during diapause, they must rely entirely on stored energy-rich molecules. Naturally, in the absence of food intake, body weight declines—typically recorded as a reduction in wet weight, though it likely also reflects a decrease in dry weight. For instance, from February to June, diapausing larvae of the chestnut weevil (*Curculio elephas*) lose about 29% of their body weight [7, 10, 11, 28].

Circadian Rhythms and Photoperiodic Regulation of Diapause in Insects

Insects exhibit daily rhythms that regulate essential behaviors such as feeding, movement, sleep, mating, and egg-laying. These rhythms are controlled by internal biological clocks known as circadian clocks, which function on a cycle of approximately 24 hours (sometimes slightly longer or shorter). Remarkably, an insect's biological rhythm persists even when it is kept in constant darkness—a phenomenon known as "free-running." A free-running rhythm is a type of biological rhythm that continues to operate in the absence of external environmental cues, such as light or temperature. It reflects the natural period of an animal's internal biological clock when it is not synchronized (entrained) by any external signals. When regular light-dark cycles resume, the circadian rhythm quickly realigns with the 24-hour day. This confirms that the rhythm is endogenously generated and not solely a reaction to environmental cues [1, 9, 29-30].

The most reliable natural signal for tracking seasons is daylength, or the amount of daylight in a 24-hour cycle. This process is called photoperiodism—it refers to how insects use changes in day length to time important events like diapause and migration [1, 9, 29-30]. Day length acts as a token stimulus: it is not harmful, but it reliably signals that unfavorable conditions—such as cold weather—are approaching. In contrast, temperature fluctuates unpredictably from day to day, especially in early autumn, making it a less dependable seasonal cue. Nevertheless, factors like temperature, rainfall, and food quality can act as additional cues for diapause induction or, in some species, even replace photoperiodic signals [1, 9, 29-30].

In the daily cycle of light and darkness, the dark period (known as the scotophase) is usually more critical for measuring day length than the light period (photophase). Light (during the photophase) and darkness (during the scotophase) function as Zeitgebers, or "time-givers"—external cues that synchronize the internal clock with the environment [1, 9, 29-30]. Another key property of circadian clocks is their temperature compensation: the rhythm remains consistent despite fluctuations in external temperature—a remarkable adaptation in ectothermic animals like insects [1, 9, 29-30].

Diapause initiation and termination are strongly influenced by environmental cues, particularly photoperiod (day length) and degree-day accumulation (a measure combining time and temperature: degrees above a threshold temperature multiplied by the number of days). These cues are processed by the brain, which releases neurochemical signals that initiate diapause [11].

Specialized photoreceptors in the insect body detect light-dark cycles. These receptors regulate genes that interpret light duration, producing proteins that trigger diapause onset. Photoperiod influences gene activation, while temperature modulates expression of key circadian "clock" mRNAs, such as *cryptochrome (cry)*, *clock (clk)*, *period (per)*, and *timeless (tim)*. These genes also govern daily biological rhythms in insects. Their expression changes predictably as days grow shorter and colder [1, 9, 29-30].

Genetic and Neuroendocrine Pathways Linking Circadian Clock Genes to Diapause in Insects

Insects possess a specific photosensitive period — a defined window during development when they can detect daylength and respond accordingly. Signals received outside this window have no effect. This photosensitive period can be extremely brief, lasting as little as a single day in species like the phantom midge (*Chaoborus americanus*), or it may extend across multiple days and developmental stages [1, 9, 29-30].

Insects detect photoperiodic signals using photoreceptors located either in the brain (a process called extraretinal photoreception), via their compound eyes (in adults) or stemmata (in larvae). In some species, both pathways are involved. However, ocelli (simple eyes) are not major contributors to photoperiod detection in insects [1-3, 31].

The insect brain plays a dual role: it not only receives seasonal cues like daylength but also stores the program that regulates diapause. For instance, in the tobacco hornworm (*Manduca sexta*), researchers transplanted brains between individuals. Brains from diapause-programmed insects induced diapause in their new hosts, whereas brains from non-diapause insects did not induce diapause in their new hosts. These results demonstrate that the brain houses both the environmental signal and the developmental response [1, 32].

To respond to light, insects require photopigments that absorb light. Two main types are involved: cryptochrome (CRY) (a blue-light sensitive protein) and carotenoid-based pigments (respond to orange-red light and are associated with a protein called opsin) [1, 9].

In fruit flies (*Drosophila melanogaster*), the CRY1 protein responds to light by binding to a circadian clock protein called TIMELESS (TIM). Light exposure leads to the breakdown of both proteins, helping reset the biological clock each day [1, 9, 29-30].

In some insect species, individuals cannot enter diapause if their diet lacks carotenoids. This has been observed in certain mites, silkworm embryos (*Bombyx mori*), and cabbage butterfly pupae (*Pieris brassicae*). When carotenoids are reintroduced into diet, their ability to enter diapause is restored. These pigments function alongside opsin proteins, which are present in the compound eyes of the insects and respond to ultraviolet, blue, and green light [9, 29-30, 33].

In the cricket *Modicogryllus siamensis*, all three opsin proteins appear to play a role in sensing daylength. However, ultraviolet and blue-sensitive opsins have a stronger effect under long-day conditions. Under short-day conditions, only the ultraviolet-sensitive opsin is significant. These opsins are concentrated in the dorsal rim area of the compound eye, making it a likely site for daylength sensing and diapause regulation [34-36]. A specialized opsin protein called bocereopsin has been discovered in the brain of *Bombyx mori*. It responds to long-wavelength light and may also contribute to diapause control [34-36].

Genetic studies show that the circadian clock (which controls daily rhythms like feeding) and the photoperiodic response (such as diapause) are genetically distinct, though likely interconnected. For example, in the fruit flies *Drosophila melanogaster*, *Drosophila littoralis*, and the mosquito *Wyeomyia smithii*, researchers have shown that circadian and photoperiodic clocks can evolve independently [9, 30]. Although circadian and photoperiodic clocks are not identical, they are not entirely independent either. They appear to cooperate in generating seasonal responses. Many of the same clock genes—such as *period*, *timeless*, and *cryptochrome*—are involved in both daily and seasonal timekeeping, although the precise mechanisms underlying their interaction remain unclear [9, 29-30].

In *Drosophila melanogaster*, a well-studied model dipteran insect, the proteins CLOCK (CLK) and CYCLE (CYC) act as positive regulators of the circadian rhythm. Inside the cell nucleus, they bind to a DNA sequence called the E-box on the period (per) and timeless (tim) genes, helping recruit RNA polymerase and thereby increasing the production of *per* and *tim* mRNAs (Figure 1). These mRNAs are then translated into PERIOD and TIMELESS proteins in the cytoplasm. PERIOD and TIMELESS proteins form a heterodimer and return to the nucleus, where PER suppresses the transcription of both *per* and *tim* genes by inhibiting CLK and CYC activity. Light influences circadian clock through a protein called CRYPTOCHROME1 (CRY1). Upon light exposure, CRY1 binds to TIM, and both proteins are degraded, effectively resetting the clock [9, 29-30, 37].

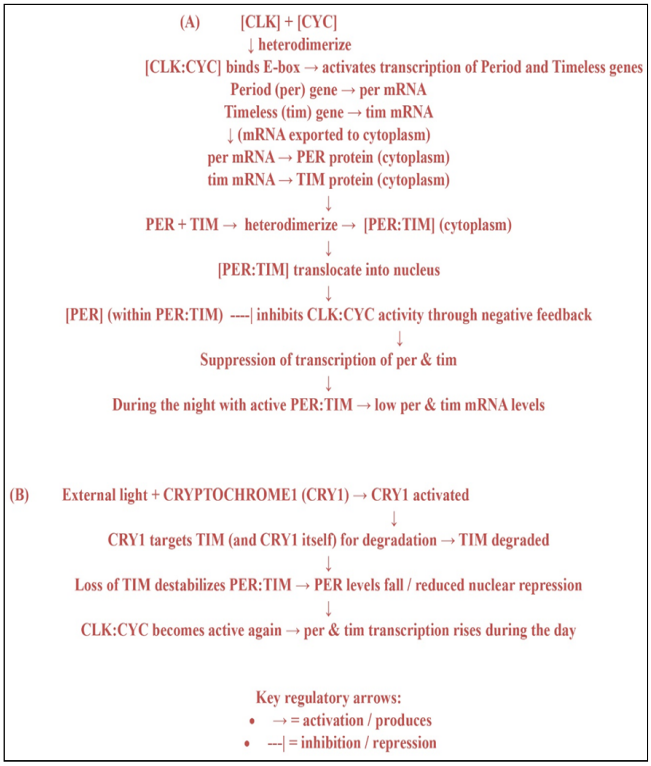

Fig 1: (A) In *Drosophila melanogaster*, the circadian rhythm is governed by a molecular feedback loop within specialized clock cells. Inside the nucleus, two proteins—CLOCK (CLK) and CYCLE (CYC)—form a heterodimer that binds to specific DNA sequences known as E-boxes located in the promoter regions of the *period (per)* and *timeless (tim)* genes. This binding activates the transcription of these genes. The resulting PER and TIM proteins then associate in the cytoplasm to form a PER–TIM complex, which subsequently translocates into the nucleus. Once inside, PER inhibits the CLK: CYC complex, thereby suppressing further transcription of the *per* and *tim* genes. (B) Exposure to light activates the protein CRYPTOCHROME1 (CRY1), which promotes the degradation of TIM and itself. Consequently, during daylight—when TIM levels are low—the CLK: CYC complex remains active, leading to high *per* and *tim* mRNA levels. During the night, the accumulation of PER–TIM complexes inhibits CLK: CYC activity, reducing the expression of the *per* and *tim* genes.

In most other insects, however, the circadian mechanism functions somewhat differently. These species possess a light-insensitive CRYPTOCHROME2 (CRY2) protein that serves as the primary negative regulator of circadian gene transcription. In this system, the PER protein facilitates the nuclear entry of CRY2, while the TIM protein plays a stabilizing role, maintaining the structural integrity of both PER and CRY2 proteins.

Researchers discovered a variant of the cryptochrome proteins, CRYPTOCHROME2 (CRY2), in the monarch butterfly (*Danaus plexippus*). Unlike CRY1, CRY2 does not respond to light. Instead, it regulates circadian gene expression by acting as a potent repressor of CLK and CYC proteins. In many insects (excluding *Drosophila* and some related flies), CRY2 — rather than PER — is likely the primary inhibitor in the circadian clock system [9, 29-30, 37].

In crickets (*Gryllus bimaculatus*), both CRY1 and CRY2 proteins are found in the eyes and contribute to light-based entrainment of circadian rhythms. They form a feedback loop that does not involve PER or TIM and is regulated by another protein, C-FOS, which helps reset the circadian clock [9, 29-30, 37].

Genes like *per* and *tim* have been identified in many insect species, and researchers are actively investigating their roles in diapause. In *Drosophila melanogaster*, flies that lack the period (per) gene show no circadian activity (i.e., they exhibit arrhythmic behavior) but can still enter diapause. This indicates that the period gene is not essential for the decision to enter diapause, although these flies display a slight shift in the critical day length required to trigger it [9, 29-30, 37].

In contrast, fruit flies lacking the timeless (tim) gene can still enter diapause, but they lose their sensitivity to day length. As a result, some individuals enter diapause even when environmental conditions do not warrant it. This suggests that tim plays a crucial role in measuring photoperiod, which is vital for the induction of diapause in *Drosophila melanogaster* [9, 29-30, 37].

In *D. melanogaster*, a protein named EYES ABSENT (EYA) accumulates during cold, short-day conditions, and TIM may help stabilize EYA, influencing the decision to enter diapause. While EYA is well-known for its role in eye development, it may also contribute to seasonal timing — a function also observed in mammals such as sheep [37-38].

In another fly species, *Chymomyza costata*, larval diapause is regulated by day length. In a mutant strain that does not respond to photoperiod, the tim gene has a deletion and lacks key regulatory sequences, including the E-box promoter. This leads to low TIM protein levels and a failure of the mutant flies to enter diapause. In wild-type *Chymomyza costata* larvae, tim shows strong cycling in short days, while this rhythm weakens in long days. Notably, in the wild-type larvae, brains exhibit two TIM-positive neurons per hemisphere, which are absent in diapause-deficient mutants. In *Chymomyza costata*, knocking down the tim gene in wild-type larvae prevents them from entering diapause, even under short day lengths that would normally induce it. Another gene called fac*et al*so appears to play a critical role in diapause in *C. costata*. A mutation in this gene abolishes *C. costata's ability to enter* diapause, suggesting a potential connection between tim and facet in *the insect's* photoperiodic response [37, 39].

In the onion maggot (*Delia antiqua*), expression levels of per, tim, and a gene called takeout rise in the head just before the onset of pupal diapause. Similarly, in adult females of *Drosophila virilis*, expression of the per gene is elevated during diapause. In the migratory locust (*Locusta migratoria*), females that lay diapausing eggs show lower expression of the takeout gene compared to females producing non-diapausing eggs. Since takeout protein acts downstream of the circadian clock, this further supports a link between clock genes and diapause-related traits [37-40].

In monarch butterflies, strong evidence indicates that vitamin A-related pathways act downstream of the circadian clock. These pathways involve genes responsible for absorbing carotenoids and converting them into retinal, a key light-sensing organic molecule. When this pathway is blocked, insects lose their ability to respond to photoperiod, even though their internal clocks remain functional. Moreover, inactivation of circadian genes such as clock, bmal1, or cry2 halts the expression of vitamin A pathway genes and leads to a loss of photoperiodic responsiveness in monarchs. In monarch butterflies, loss-of-function mutations in the Clk and Bmal1 genes lead to increased egg-laying under short days. In contrast, mutants of the cry2 gene show reduced egg production under long days — a behavior typical of diapause [9, 29-30, 41].

In the linden bug *Pyrrhocoris apterus*, silencing the clock genes cyc and Clk during the light-sensitive nymphal stage leads to adults exhibiting diapause-like traits, a finding consistent with results from the bean bug. In the cabbage beetle *Colaphellus bowringi*, RNAi targeting per and tim genes does not alter whether females enter diapause, but these females fail to accumulate the fat reserves typically required during diapause. In *Nasonia vitripennis*, short-day conditions prompt females to lay diapause eggs. However, this response is lost when the period gene is knocked down, indicating its role in photoperiodic regulation. Interestingly, low temperatures still induce diapause in offspring from per knockdown females, suggesting that the per gene is specific to photoperiodic input rather than diapause capability itself. In *Antheraea pernyi*, exposure to long days terminates pupal diapause, but RNAi targeting cyc, Clk, or the per gene disrupts this response, allowing adult moths to emerge from pupal cases even under short-day conditions [9, 29-30, 37].

Taken together, these findings indicate that a functional circadian clock is often essential for proper diapause regulation. However, exceptions exist. For example, in *P. apterus*, RNAi targeting per, cry, Clk, cyc, and pdp1 genes does not directly influence diapause, although gene expression in the gut is still altered [29-30].

In the mosquito *Culex pipiens*, the expression of clock, per, tim, and vrille genes is suppressed during the pupal stage, when the insect is being programmed for adult diapause. However, these changes are absent in diapausing adults. RNAi studies show that silencing per, tim, and cry2 genes, causes *Culex* mosquitoes that would otherwise enter diapause to skip it. Conversely, knocking down pdf (pigment-dispersing factor), a gene that acts downstream of the clock gene, causes long-day females to display diapause-like traits [29-30].

The genes vrille (vri) and Pdp1, which regulate the Clk gene in a secondary feedback loop, also influence diapause in *C. pipiens*. Silencing the vri gene yields inconsistent effects on egg follicle development but does not impact fat storage in *C. pipiens*. In contrast, knockdown of the Pdp1 gene blocks fat accumulation without affecting follicle development, indicating that these genes modulate distinct aspects of the diapause phenotype. Interestingly, Pdp1 shows oscillatory expression only under long days and remains constantly high in short-day diapausing females. Two genes that act downstream of the clock—takeout (to) and Nocturnin (Noc)—display similar expression patterns. Knocking down Pdp1 leads to increased expression of the takeout and Noc genes. Noc may promote fatty acid synthesis, while takeout supports sugar feeding, enhances stress survival, and

suppresses juvenile hormone (JH) signaling. Notably, the takeout gene is also regulated by FoxO (Forkhead Box O), a key transcription factor involved in diapause-related pathways in *C. pipiens* [29-30, 37, 42].

The mosquito *Culex molestus*, a close relative of *C. pipiens* that does not undergo diapause, carries a 9-base pair deletion in the domino (dom) gene. Studies in *Drosophila* have shown that the DOMINO protein regulates both period and timeless genes, suggesting that this mutation in domino gene may explain the absence of diapause in *C. molestus* [29-30, 43].

In *Manduca sexta*, the duration of diapause depends on the number of short days (periods of short daylight) experienced by the larvae. When larvae encounter many short days early in development (early autumn), diapause is relatively brief. In contrast, if short days occur late in development (late summer), the resulting diapause is prolonged. This mechanism allows the insect to adjust diapause duration in response to seasonal cues. Remarkably, scientists have demonstrated that this seasonal memory can be transferred through brain transplantation, confirming that the brain stores this timing information [32].

In the silkmoth *Antheraea pernyi*, light-sensitive cells are located in the dorsolateral region of the brain. This area also contains neurosecretory cells that release PTTH (prothoracicotropic hormone), a key regulator of diapause. Similarly, in the tobacco hornworm *Manduca sexta*, removing specific neurosecretory cells prevents diapause, indicating that these cells are involved in sensing day length. These neurosecretory cells are located near brain cells that produce circadian clock proteins such as PERIOD, facilitating efficient communication between light-sensitive inputs and hormone-releasing centers [29-30, 32, 43].

Likewise, in the blowfly *Protophormia terraenovae*, removing neurons that produce pigment-dispersing factor (PDF) disrupt the ability to discriminate day length and results in reproductive diapause regardless of photoperiod. These findings are consistent with earlier RNAi studies in *C. pipiens*, in which PDF protein was identified as a key downstream effector in the diapause pathway. In the silkworm *Bombyx mori*, PDF receptors are present in the prothoracic gland, and PDF has been shown to stimulate ecdysone synthesis, further linking the circadian clock to hormonal regulation of diapause [31, 42-44].

In the migratory locust *Locusta migratoria*, females raised under short-day conditions produce eggs that enter diapause, whereas long-day conditions result in non-diapause eggs. A gene expression study of their central nervous system revealed over 600 genes that were differentially expressed depending on day length—360 upregulated and 250 downregulated. One of these, the *takeout* gene, is regulated by the circadian clock. Suppressing *takeout* using RNA interference (RNAi) has no effect under long-day conditions, but in short-day locusts, it increases the incidence of diapause in offspring from 71% to 94% [40, 45].

The *couch potato (cpo)* gene, which encodes an RNA-binding protein, may play a role in diapause regulation. It is expressed in multiple tissues, including the brain and larval ring gland of *Drosophila melanogaster*. In the flesh fly *Sarcophaga crassipalpis*, which undergoes pupal diapause, *cpo* expression is downregulated during diapause [24]. In the mosquito *Culex pipiens*, *cpo* transcript levels are initially similar between diapausing and non-diapausing females at eclosion (the emergence of an adult insect from its pupal case). However, a week later, diapausing females show a significant increase in cpo expression. After diapause ends—either naturally or following juvenile hormone treatment—*cpo* expression declines [42].

Although the complete molecular pathways linking clock genes to the diapause phenotype in insects have yet to be fully elucidated, emerging evidence suggests that core clock genes influence diapause-related traits through downstream effectors such as FoxO, microRNAs, and JH signaling.

Mechanisms of Cell Cycle Arrest During Insect Diapause

A striking feature of diapause is the arrest of the cell cycle, meaning that cells stop dividing. However, different insect species halt the cell cycle at different stages. The end result is the same — development is halted — but the underlying mechanisms vary across insect species [7, 10]. For example, in the silkworm (*Bombyx mori*), embryonic cells stop at the G2 phase of the cell cycle. This has been confirmed by flow cytometry and analysis of the expression of key regulatory genes such as Cdc2 and Cdc25 [44].

In contrast, the brain cells of diapausing pupae of the flesh fly *(Sarcophaga crassipalpis)* arrest at the G0/G1 phase. This correlates with a reduction in the expression of the gene pcna, which normally helps cells transition from G1 to S phase. Interestingly, other genes like cyclin E, p21, and p53 do not appear to be affected in this species, suggesting that PCNA protein may play a special role in initiating diapause. Additionally, increased phosphorylation of CDK1, a key cell cycle regulator, has been observed in the brain during diapause and may contribute to keeping the cells in a resting state [24]. In the tobacco hornworm (*Manduca sexta*), cells that will eventually form the eyes (the optic lobe anlagen) also arrest at the G2 phase during diapause [7, 10].

Insects that undergo larval diapause, such as the fly *Chymomyza costata* and the jewel wasp *Nasonia vitripennis*, display mixed patterns of cell cycle arrest. In both species, most brain cells halt at G0/G1, but 13–20% of cells arrest at G2 during diapause, showing a mixed arrest pattern. In *C. costata*, the expression of many cell cycle-related genes — including cyclin B, cyclin E, and Cdk1, Cdk2, and Cdk7 — is also reduced during diapause. These genes normally regulate key transitions in the cell cycle (such as G1/S and G2/M), so their downregulation further enforces the developmental halt [7-10].

In *Daphnia*, a freshwater crustacean, embryos enter diapause when they reach approximately 3,500 cells. At that point, cell division halts. Interestingly, these embryos grow more slowly prior to diapause, with a cell division rate that is 2.5 times lower than that of their non-diapausing counterparts [7-10].

Roles of Heat Shock Proteins and Antioxidants in Stress Tolerance Mechanisms During Insect Diapause

A major stress response observed during diapause in insects is the increased production of heat shock proteins (Hsps), also known as stress proteins. These proteins help protect cells under stressful conditions. The Hsp family includes small Hsps (20–30 kDa), Hsp60, Hsp70, and Hsp90. While some Hsps are only produced under stress (heat-inducible), others—such as heat shock cognate 70 (Hsc70)—are constitutively expressed under nonstressed conditions [4-5, 7-10, 45].

Interestingly, Hsp production during diapause occurs even at mild temperatures that would not normally trigger a stress response. Unlike the classic heat shock response—where the synthesis of most other proteins is suppressed—Hsps are synthesized alongside many other proteins during diapause [7-10, 45].

In the flesh fly *Sarcophaga crassipalpis*, pupal diapause is marked by increased expression of two genes (*hsp23* and *hsp70*) encoding heat shock proteins, along with decreased expression of the *pcna* gene, which is important for cell division. However, these gene expression changes do not occur when the insect transitions from diapause to post-diapause dormancy, indicating that this genetic pattern is specific to diapause itself. In *Sarcophaga crassipalpis*, RNA interference (RNAi) targeting Hsp23 or Hsp70 does not influence either the decision to enter diapause or the duration of the diapause state. In brine shrimp (*Artemia franciscana*), knocking down Hsp40 prevents more than half of the nauplii (larvae) from entering diapause. This is the first known case in which a heat shock protein directly affects diapause initiation [7, 8, 10, 45].

Another key stress response during diapause is the antioxidant response, which protects insects from damage caused by reactive oxygen species (ROS). ROS include superoxide anions, hydroxyl radicals, alkoxyl radicals, peroxyl radicals, and hydrogen peroxide. While low levels of ROS can function as signaling molecules, excessive ROS can damage proteins, lipids, and DNA [4-5, 7-10].

To counter ROS-induced damage, insects rely on both enzymatic and non-enzymatic antioxidants. These include scavengers such as glutathione and iron-binding proteins like ferritin, as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase. For example, tocopherols (vitamin E), which are known antioxidants, accumulate in the cell membranes of diapausing adults of the linden bug (*Pyrrhocoris apterus*) during winter. Other compounds like carotenoids and hemocyanin also contribute to ROS defense. These biochemical defenses, along with strategies like discontinuous gas exchange, help minimize oxidative damage during diapause [4-5, 7-10].

In young diapausing female *Culex pipiens*, levels of antioxidant enzymes such as catalase and SOD-2 are significantly higher than in non-diapausing females. When the gene encoding catalase is knocked down using RNAi, the result is increased ovarian damage and reduced lifespan in diapausing female insects. In contrast, suppression of the sod-2 gene does not produce the same detrimental effects. Both genes are part of a signaling pathway regulated by the FoxO transcription factor [4-5, 7-10].

In the migratory locust (*Locusta migratoria*), reducing the levels of peroxiredoxin 6 (Prx6) also leads to decreased levels of catalase and SOD. This suggests that Prx6 plays a key role in regulating the antioxidant response. Interestingly, silencing the Prx6 gene increases the phosphorylation of FoxO and reduces the occurrence of embryonic diapause, indicating a possible connection between ROS, insulin signaling, and diapause regulation [46].

Diapausing larvae of the mountain pine beetle (*Dendroctonus ponderosae*) also exhibit increased levels of glutathione peroxidase and the ROS scavenger ferritin. In the cotton bollworm (*Helicoverpa armigera*), both SOD and glutathione-S-transferase (GST) levels rise during pupal diapause. Similarly, in the solitary bee (*Megachile rotundata*) and the parasitoid wasp (*Trichogramma dendrolimi*), the expression of peroxidase and GST genes increases during diapause. Elevated GST levels have also been observed in diapausing adults of *Drosophila suzukii* and *Culex pipiens pallens* [4-5, 7-10].

Additionally, cytochrome P450 enzymes—a group known for oxidizing a wide variety of substrates—show increased activity during diapause in insects. This has been observed in the cricket (*Allonemobius socius*), the migratory locust, and the silk moth (*Antheraea yamamai*), all during embryonic diapause, and in other species such as *Bombyx mori*, *Trichogramma dendrolimi*, and *Bactrocera minax* during various diapause stages [4-5, 7-10].

Molecular and Hormonal Regulation of Larval Diapause in Insects

In many insect species, larval diapause is maintained by high levels of juvenile hormone (JH) and low levels of ecdysteroids (molting hormones). In many insects, the prothoracic glands either fail to produce sufficient ecdysteroids or become unresponsive owing to the dominant action of JH during diapause, which blocks further development [4-5, 7-11].

A key hormone in reactivating development after completion of diapause is prothoracicotropic hormone (PTTH), which is synthesized in specialized neurosecretory cells in the insect brain. PTTH secretion typically occurs only after the brain experiences prolonged exposure to cold. Without PTTH, the prothoracic glands remain inactive; no ecdysone (a major ecdysteroid) is produced, and the insect cannot progress to the next developmental stage [4-5, 7-11].

Antheraea yamamai, a lepidopteran insect, displays a distinctive form of diapause at the pharate (pre-emerged) first-instar larval stage. In this species, diapause can be effectively terminated by KK-42, a compound that inhibits ecdysteroid synthesis. A specific pentapeptide, Yamamarin, particularly in its palmitoyl-conjugated form, has been identified in the diapausing larvae of *Antheraea yamamai*. Yamamarin can reversibly arrest the cell cycle and reduce metabolic activity, inducing a diapause-like state. Remarkably, Yamamarin also inhibits the growth of mammalian cells in addition to insect cells [47].

In the mosquito *Aedes albopictus*, pharate first-instar larval diapause is triggered by low levels of juvenile hormone (JH). While ecdysteroid levels remain similar in diapausing and non-diapausing embryos, JH III levels become significantly lower at the onset of diapause. This observation is reinforced by gene expression data, which have shown that genes involved in JH synthesis are downregulated during larval diapause in *Aedes albopictus*, whereas genes normally activated by JH are also expressed at lower levels. Additionally, a gene encoding JH esterase, an enzyme that degrades JH, is upregulated at the beginning of larval diapause and stays active throughout [4-5, 7-11].

These findings support a mechanism for sustaining low JH levels during diapause. Furthermore, treatment with a JH analog (pyriproxyfen) can successfully terminate larval diapause in *Aedes albopictus*, reinforcing the conclusion that suppressed JH signaling is key to diapause maintenance [4-5, 7-11].

In the silkworm *Bombyx mori*, whether offspring enter diapause depends on environmental signals such as long daylight hours and high temperatures experienced by the mother during her embryonic or early larval stages. If she

receives these environmental cues early in life, she will later produce a special neuropeptide called diapause hormone (DH) as an adult. This hormone acts on her ovaries and determines whether her eggs will enter diapause [7, 11, 35, 44].

Diapause hormone (DH) is a 24-amino acid peptide belonging to the FXPRL-amide family. In *Bombyx mori*, when the genes encoding DH or its receptor are knocked out using TALEN-based gene editing, females lay only non-diapause eggs, demonstrating that DH is essential for inducing diapause [7, 11, 35, 44].

Neurotransmitter-Mediated Regulation of Diapause in Insects

Neurotransmitters like dopamine and serotonin are strongly implicated in regulating pupal diapause in insects. Elevated brain dopamine levels are crucial for initiating diapause in insects. This may be due to dopamine's ability to inhibit PTTH release, as observed in several species: larvae of the fly *Chymomyza costata*, pupae of moths like *Mamestra brassicae* and *Antheraea pernyi*, butterflies such as *Pieris brassicae*, and adults of *Leptidea sinapis*. Interestingly, in *A. pernyi*, brain dopamine levels drop just before diapause ends [4-5, 7-11, 48].

Further supporting dopamine's role, experiments show that activating D2-like dopamine receptors in *A. pernyi* delays adult emergence under long-day conditions. Conversely, blocking these receptors terminates diapause, likely because inhibition of adenylate cyclase prevents the production of cyclic AMP (cAMP)—a key intracellular signaling molecule. One of the pieces of evidence comes from *M. brassicae*, where feeding L-DOPA (a dopamine precursor) to larvae reared under long days (which normally prevent diapause) induced a diapause-like state in the resulting pupae [48].

Both short daylengths and L-DOPA feeding upregulate expression of a gene associated with RACK (receptor for activated protein kinase C), a component of the PKC signaling pathway. This pathway may help maintain high dopamine levels that suppress PTTH release, thereby supporting diapause maintenance [48].

Elevated levels of serotonin and dopamine promote adult diapause, whereas reducing these signals decreases the likelihood of diapause. Serotonin appears to inhibit ILP production and release, contributing to the diapause state. When dopamine receptor 1 (*DopR1*) is silenced using RNAi in the CA and fat body *of Drosophila melanogaster*, the incidence of diapause decreases, indicating that *DopR1* signaling is involved in the regulation of adult diapause [48].

A specific serotonin receptor, 5HTRβ, expressed on PTTH-producing cells in *A. pernyi*, appears to inhibit PTTH synthesis or release. When this receptor is silenced via RNA interference (RNAi), pupal diapause prematurely terminates, and PTTH accumulates within the cells. This strongly suggests that 5HTRβ helps maintain diapause by regulating PTTH output [48].

Roles of PTTH, Ecdysteroids, and Signal Transduction Pathways in Regulating Insect Diapause

Not only is PTTH absent or unreleased during diapause in insects, but its target—the prothoracic gland (PG)—also becomes unresponsive. Experiments show that PGs from diapausing insects fail to respond to PTTH stimulation. This phenomenon has been documented in larval diapause of the blow fly (*Calliphora vicina*) and the corn borer (*Ostrinia nubilalis*), as well as in pupal diapause of *Manduca sexta* (tobacco hornworm) and *Sarcophaga argyrostoma* (flesh fly). In *S. argyrostoma*, the PG ceases ecdysone production—a steroid hormone critical for development—just 1–2 days after diapause begins. In *C. vicina*, this decline occurs more gradually over about six days. Interestingly, even during diapause, the PG retains some capacity to produce ecdysone. Its responsiveness can be rapidly restored: transferring diapausing larvae of blow flies from 11°C to 25°C enables the PG to resume ecdysone synthesis within a day [1-10, 30].

Another critical factor is the neuropeptide myosuppressin, which contributes to the suppression of PG at diapause onset. In *M. brassicae*, the PG becomes resistant to PTTH within one day after pupation, coinciding with a spike in myosuppressin levels in hemolymph. Laboratory studies demonstrate that myosuppressin can block PTTH's action on the PG, even when PTTH is present. This shows that during diapause, not only is PTTH absent, but the PG is actively suppressed to prevent activation [1, 4-5, 7-10, 30].

Whether the hormone PTTH is not synthesized or merely not released during diapause depends on the insect species. However, the final outcome remains the same: the synthesis of ecdysteroids (molting hormones) is blocked [4-5, 7-11].

Numerous studies have shown that during diapause—whether in the larval or pupal stage—ecdysteroid levels are extremely low or undetectable. These hormone levels rise again once diapause ends. This pattern has been observed in species such as the European corn borer (*Ostrinia nubilalis*), bamboo borer (*Omphisa fuscidentalis*), seabuckthorn carpenterworm (*Holcocerus hippophaecolus*), yellow-spotted longicorn beetle (*Psacothea hilaris*), Bertha armyworm (*Mamestra configurata*), cabbage moth (*Mamestra brassicae*), tobacco hornworm (*Manduca sexta*), and flesh flies (*Sarcophaga argyrostoma* and *S. peregrina*), among others [1-2, 7-11].

A key indication that ecdysteroids are essential for diapause termination comes from experiments in which exogenous (externally supplied) ecdysteroids or similar compounds are injected into insects—resulting in diapause termination and the resumption of development. This hypothesis is supported by the results obtained from studies involving KK-42, a synthetic growth regulator that inhibits ecdysteroid production. Treatment with KK-42 in species like *Antheraea pernyi*, *Helicoverpa zea*, and *Sarcophaga crassipalpis* prolongs diapause or increases its likelihood, reinforcing the concept that low ecdysteroid levels help maintain diapause [47, 49].

The concept of shutting down the brain–prothoracic gland (PG) hormonal axis has become a widely accepted model for explaining diapause. In this model, the PG fails to produce ecdysone (a type of ecdysteroid) because it does not receive the necessary stimulatory signal—PTTH—from the brain. Cold exposure (chilling) of the brain is required to trigger PTTH release. Once released, PTTH activates the PG to produce ecdysone and terminate diapause. However, not all insects conform to this model [1-2, 7-11].

While PTTH remains a key regulator of diapause through its control over the PG, other signaling pathways also contribute to PG regulation. For example, in *Sarcophaga crassipalpis*, levels of cyclic AMP (cAMP), a common second messenger, are high in the brain and ring gland of non-diapause pupae and low in those that enter diapause. Artificially raising cAMP levels in diapause-destined pupae

can prevent diapause, suggesting that cAMP facilitates PTTH signaling [1-2, 7-11].

However, injecting cAMP together with ecdysteroids slows development rather than accelerating it. In contrast, cyclic GMP (cGMP)—another signaling molecule that cannot prevent diapause on its own—can terminate diapause and is even more effective when combined with ecdysteroids. The reason cGMP can terminate diapause, while cAMP cannot, remains unclear [1-2, 7-11].

One important part of the signaling pathway that leads to the production of ecdysone—a steroid hormone necessary for insect development—is the enzyme ERK (extracellular signal-regulated kinase), which belongs to the MAPK (mitogen-activated protein kinase) family. In the flesh fly *Sarcophaga crassipalpis*, when pupal diapause ends—either through exposure to high temperature or a chemical like hexane—one of the earliest detectable changes is the activation (through phosphorylation) of ERK. Interestingly, injecting ecdysteroids (the active form of ecdysone) does not trigger ERK activation, nor is ERK activated in the ring gland, where ecdysone is produced [1, 7-11, 50].

The PTTH receptor in the prothoracic gland (PG) is a tyrosine kinase known as Torso. When PTTH binds to this receptor, it activates ERK, which then helps stimulate ecdysone production. Therefore, although one might expect ERK to be active in the ring gland when diapause ends, it is actually activated only in the brain, fat body, and epidermis—not in the ring gland. Clearly, both PTTH and ERK play major roles in restarting development after diapause ends [1, 7-11, 50].

Another factor that may control the timing of diapause termination is the presence of the receptor for 20-hydroxyecdysone (the active form of ecdysone). This receptor is a protein complex composed of two parts: EcR (ecdysone receptor) and USP (Ultraspiracle). When 20-hydroxyecdysone binds to this receptor, the complex can attach to specific DNA sequences called ecdysone response elements to activate genes required for development. Without this receptor complex, cells cannot respond to the hormone; thus, the presence of the receptor is essential [1, 7-10, 51].

In *Sarcophaga crassipalpis*, the gene for EcR is active throughout pupal diapause, but the USP gene is switched off at the onset of diapause and remains inactive during its middle phase. It is reactivated during late diapause, just as pupae become more responsive to ecdysteroid injections. This activation of USP may be a key step in preparing the insect to end diapause [7-10, 24].

In the second-instar larvae of the spruce budworm (*Choristoneura fumiferana*), both EcR and USP genes remain active throughout diapause, but several genes that respond to ecdysone only become active at the end of diapause. In *Manduca sexta*, EcR expression disappears at the onset of diapause and returns when diapause ends. Similar patterns have been observed in other insects such as the midge *Chironomus tetans*, the bamboo borer *Omphisa fuscidentalis*, and the seabuckthorn carpenterworm *Holcocerus hippophaecolus* [24, 51].

One set of experiments highlights a feedback mechanism between the fat body and the brain that helps control the termination of pupal diapause in the bollworm *Helicoverpa armigera*. During diapause, the fat body shows a strong reduction in gene activity, resulting in lower levels of tricarboxylic acid (TCA) cycle intermediates in the insect's hemolymph (blood). When diapause ends, the resumption of metabolism in the fat body releases large amounts of these TCA intermediates. These molecules act on the brain, triggering the release of prothoracicotropic hormone (PTTH), which then stimulates the production of ecdysteroids—hormones that drive development and metamorphosis. This hypothesis is further supported by the finding that injecting TCA intermediates or their precursors into *Helicoverpa armigera* can also break pupal diapause [52].

In the gypsy moth (*Lymantria dispar*), diapause occurs at the stage of a pharate first-instar larva, a fully formed larva still enclosed within the eggshell. When diapause ends, the larva consumes the remaining yolk, breaks through the eggshell, and begins its active life. There is strong evidence that diapause in *Lymantria dispar* is both initiated and maintained by elevated levels of ecdysteroids. An imidazole compound called KK-42, which inhibits ecdysteroid biosynthesis, prevents diapause in *L. dispar*. However, administering ecdysteroids to these KK-42-treated embryos can restore the diapause state. Similarly, after a chilling period, simply warming the eggs can terminate diapause—unless ecdysteroids are added, which then halts this resumption of development. Studies on a mutant strain of *L. dispar* that normally does not enter diapause show that artificially increasing ecdysteroid levels reinstates the diapause response. The production of hemolin, a 55-kDa immune protein and a reliable gut marker of diapause in this species, is disrupted when the activities of the prothoracic gland are suppressed; however, adding ecdysteroids or co-culturing the gut with an active prothoracic gland induces hemolin biosynthesis. This body of evidence strongly suggests that high ecdysteroid levels drive and maintain diapause in *L. dispar* [53].

Thus, although different insect species show varied expression patterns of the 20-hydroxyecdysone receptor gene during diapause, the presence—or absence—of these receptor proteins appears to be a crucial element in how insects regulate the entry into and the exit from larval and pupal diapause.

Roles of Juvenile Hormone (JH) in Regulating Insect Diapause

Some insects continue to molt even during diapause. Examples include the southwestern corn borer (*Diatraea grandiosella*), the Mediterranean corn borer (*Sesamia nonagrioides*), rice and maize stem borers (*Chilo suppressalis*, *Busseola fusca*), and the cricket *Modicogryllus siamensis* [11].

These molts during diapause are known as stationary molts because the insects do not grow significantly in size or weight during these molts. Although molting during a dormant phase seems energetically costly, one possible benefit is the shedding of cuticles that may be contaminated with fungi or pathogens [11].

The occurrence of stationary molts during diapause suggests the continued presence of juvenile hormone (JH), an acyclic sesquiterpene. Under normal circumstances, the absence of JH allows insects to proceed to the pupal stage. During diapause, JH levels in the hemolymph remain high, and the corpora allata (CA)—the gland that produces JH—remains active. As diapause ends, the drop in JH levels permits

ecdysone release and initiates pupation [1, 11]. High JH levels have also been reported in the diapausing larvae of other species, such as the Mediterranean corn borer and the yellow-spotted longicorn beetle (*Psacothea hilaris*). In the brown planthopper (*Laodelphax striatellus*), knocking down the transcription factor FoxO reduces JH levels and shortens diapause duration, further supporting the role of JH in maintaining diapause in these insects [4-5, 7-11].

In contrast, some species do not undergo stationary molts and do not require high JH levels to sustain diapause. For example, in the European corn borer (*Ostrinia nubilalis*), the codling moth (*Cydia pomonella*), and the beet webworm (*Loxostege sticticalis*), JH levels are high at the onset of diapause but decline rapidly thereafter. Injecting these insects with ecdysteroids results in pupation rather than another molt, indicating that JH does not maintain diapause in these species. Furthermore, applying JH to non-diapausing larvae in these insects does not induce diapause, suggesting that JH is likely not involved in initiating diapause either. Interestingly, in some insects, such as the bamboo borer (*Omphisa fuscidentalis*), applying JH or its analogs can actually terminate both larval and pupal diapause [1, 4-5, 7-11].

In *Diatraea grandiosella*, applying a JH analog to non-diapausing larvae can induce a diapause-like state and lead to the accumulation of hexamerin—a storage protein in the fat body typically associated with diapause. If the corpora allata are surgically removed (allatectomy) from diapausing larvae, hexamerin fails to accumulate in the fat body. In larvae preparing for diapause, hexamerin levels peak at the onset of diapause, then gradually decline as the protein is used up, leaving only trace amounts by the end of the diapause period [7-11].

When JH is externally applied to diapausing females, such as in the beetle *Leptinotarsa decemlineata*, it may only trigger a brief period of egg-laying. In the pea aphid (*Acyrthosiphon pisum*), JH also regulates the switch to sexual reproduction in autumn, a crucial step for producing overwintering eggs in a dormant (diapause) state. Application of JH during this period prevents the aphid from switching to this reproductive mode [4-5, 7-11].

Roles of Corpora Allata (CA) in Regulating Diapause in Insects

As in larval and pupal stages, the brain continues to serve as the primary regulatory center for adult diapause, mainly by modulating the activity of the corpora allata (CA), an endocrine gland. The brain exerts control over the CA through both neural inputs and neuropeptides, such as allatotropins (which stimulate CA activity) and allatostatins (which inhibit CA activity) [1, 4-5, 7-10].

In insects programmed for diapause, the CA is typically small and inactive. Removing the CA from non-diapause (long-day) beetles induces a diapause-like state. Similarly, destruction of the CA using precocene II causes long-day beetles to enter a diapause-like condition [1, 4-5, 7-10].

Suppression of the allatotropin gene via RNA interference (RNAi) in female *Culex pipiens* that are not programmed to enter diapause also leads to a diapause-like state. However, this condition can be reversed by the application of juvenile hormone III (JH III). During natural diapause, allatotropin mRNA levels are significantly reduced, suggesting that the corresponding regulatory protein plays a role in diapause control. Finally, silencing ribosomal protein genes *S2* and *S3a* also induces diapause-like states in *Culex pipiens*, which can likewise be reversed by JH III application [4-5, 7-10].

The role of the corpora allata (CA), the gland responsible for producing juvenile hormone (JH), can differ between males and females in regulating diapause. For example, in the grasshopper *Anacridium aegyptium*, a non-functional CA in males is consistent with diapause, as evidenced by the absence of mating behavior. However, in species such as *Pyrrhocoris apterus* and *Protophormia terraenovae*, removal of the CA (a procedure known as allatectomy) has little effect on male behavior but significantly impacts females. In these species, males continue to respond to photoperiod and display mating behavior even without a CA. Nonetheless, the development of accessory glands and the production of storage proteins (hexamerins) still depend on the CA [1, 4-5, 7-11].

FoxO as a Central Regulator of Insect Diapause

Interestingly, in species like the Australian plague locust (*Chortoicetes terminifera*) and the migratory locust (*Locusta migratoria*), low ecdysteroid levels appear to trigger embryonic diapause. In these locusts, non-diapausing eggs contain approximately three times more ecdysteroids than diapausing ones; these ecdysteroids are likely of maternal origin. A similar increase in ecdysteroid levels is seen when diapause ends in *L. migratoria*, and externally administered ecdysteroids can terminate diapause. This suggests that, as in many other insect species, diapause in these locusts is induced by the *absence* of ecdysteroids. As in silkworms, the termination of embryonic diapause in *L. migratoria* is also linked to ERK pathway activation. Moreover, the diapause fate of eggs laid by *L. migratoria* females is influenced by reactive oxygen species (ROS) and insulin signaling, particularly through phosphorylation of the FoxO transcription factor [1, 4-5, 7-11].

FoxO is a key transcription factor whose activity is regulated by insulin signaling. In the presence of insulin, FoxO is inactivated through phosphorylation; in the absence of insulin, FoxO becomes active and translocates from the cytoplasm into the nucleus, where it initiates various cellular responses [1, 54-55].

A model for pupal diapause control in *Helicoverpa armigera* highlights the complex interactions among reactive oxygen species (ROS), insulin/insulin-like growth factor signaling (IIS), and epigenetic regulators (Figure 2). Under long-day conditions, high levels of insulin-like peptides (ILPs) activate the IIS pathway, which suppresses the activity of FoxO. This suppression lifts the block on

prothoracicotropic hormone (PTTH) gene expression, allowing development to proceed without entering diapause [1, 54-55].

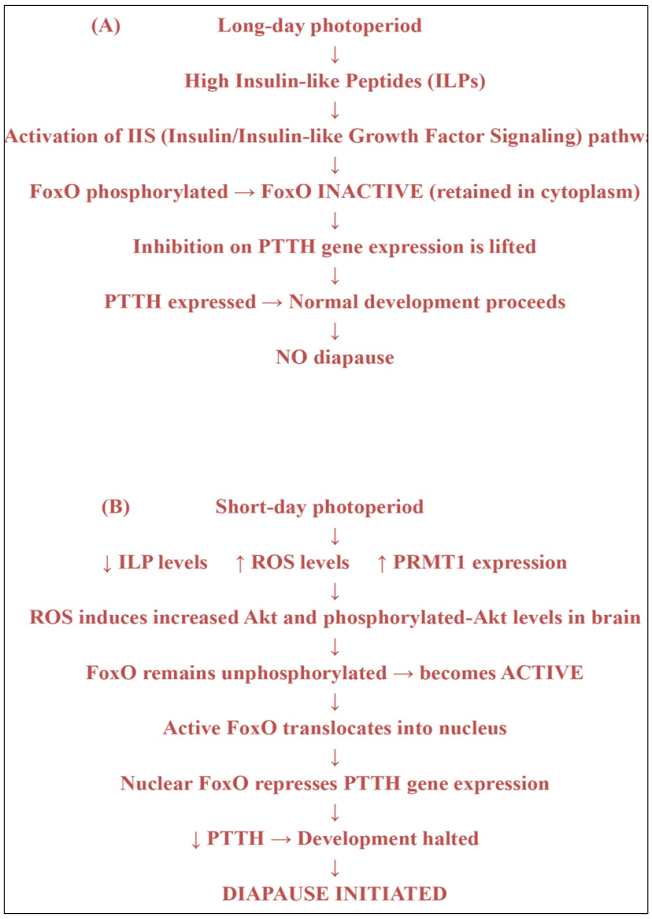

Fig 2. In *Helicoverpa armigera*, the interaction among reactive oxygen species (ROS), insulin signaling (IIS), and epigenetic regulators plays a crucial role in controlling pupal diapause. Under long-day (LD) conditions, elevated levels of insulin-like peptides (ILPs) activate the IIS pathway, leading to the phosphorylation and subsequent inactivation of the transcription factor FoxO. This suppression permits the expression of genes responsible for producing prothoracicotropic hormone (PTTH), resulting in normal, uninterrupted development into the adult stage—thus, diapause does not occur. (B) In contrast, under short-day (SD) conditions, the situation is reversed. Reduced ILP levels, together with elevated ROS and PRMT1 activity, prevent FoxO phosphorylation. Consequently, the active FoxO translocates into the nucleus, where it represses *PTTH* gene expression, halting further development and inducing pupal diapause.

In contrast, under short-day conditions, ILP levels decline while ROS levels and the expression of PRMT1 (protein arginine methyltransferase 1) increase. Increased levels of ROS induce high levels of Akt (a serine/threonine kinase) and its phosphorylated form in the insect brain (Figure 2). These changes prevent the phosphorylation of the FoxO transcription factor, allowing it to accumulate in the nucleus. Nuclear FoxO then represses the expression of the gene encoding PTTH, leading to the initiation of diapause [1, 54-55].

Another important regulator of diapause is hexokinase, the first enzyme in the glycolytic pathway. Reduced hexokinase activity slows metabolism and elevates ROS levels, both of which favor diapause. During short-day conditions, decreased ecdysone levels lead to reduced expression of the transcription factors POU and c-Myc. This further diminishes hexokinase levels, resulting in lower metabolic activity and increased ROS—hallmarks of the diapause state [7-10, 54-55].

In *Drosophila* populations that do undergo diapause, researchers have observed a reduction in insulin signaling, which is linked to the suppression of reproductive activity during diapause. FoxO, a transcription factor, becomes active when insulin signaling is low. Active FoxO induces the expression of specific genes that promote energy storage through lipid accumulation, enhance stress resistance by upregulating antioxidant enzymes such as superoxide dismutase, and suppress cell growth and reproductive processes (Figure 3) [1, 7-10, 54-55].

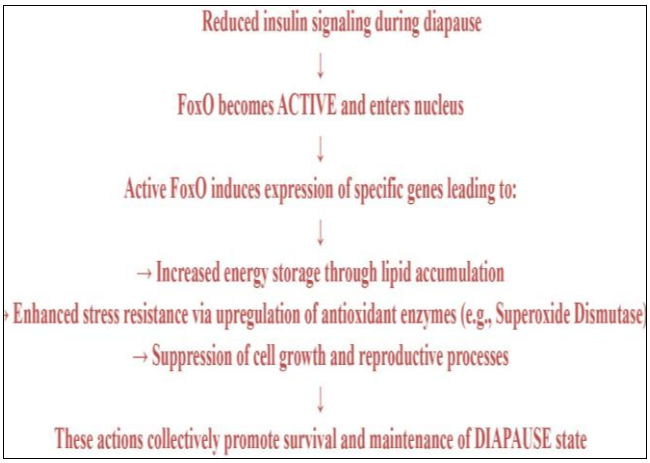

Fig 3: FoxO is a transcription factor that becomes active when insulin signaling is low. Once activated, FoxO induces the expression of genes that promote the storage of energy-rich molecules by enhancing lipid accumulation and increase stress tolerance by upregulating antioxidant enzymes such as superoxide dismutase. At the same time, FoxO activity suppresses cell growth and reproduction. In mosquitoes that enter diapause, experiments using RNA interference (RNAi) to silence the *FoxO* gene prevent the accumulation of the large fat reserves normally observed during diapause. Overall, FoxO functions as a central regulatory protein controlling the diapause state, coordinating metabolic, stress-response, and developmental pathways to ensure the insect's survival under unfavorable conditions.

Insulin signaling also plays a pivotal role in diapause regulation. When insulin-producing cells (IPCs) in the brain—responsible for producing insulin-like peptides (ILPs) such as ILP-2, ILP-3, and ILP-5—are ablated or silenced in *Drosophila melanogaster*, or when the insulin signaling pathway is blocked (e.g., via the *chico* mutation or reduced ILP levels in the hemolymph), diapause or dormancy is markedly enhanced. Conversely, increasing ILP expression or enhancing IPC activity prevents diapause. These findings suggest that reduced insulin signaling is a trigger for dormancy [7-10, 54-55].

The effects of JH on larval growth depend on the transcription factor FoxO, which is normally inactivated when insulin signaling is high. Under long-day conditions (when insects remain reproductively active), insulin signaling suppresses FoxO. In contrast, under short-day conditions (which induce diapause), reduced insulin signaling activates FoxO. In mosquitoes programmed for diapause, silencing FoxO using RNA interference (RNAi) prevents the accumulation of large fat stores typical of the

diapause state, indicating that FoxO activity is essential for diapause in insects [7-10, 54-55].

In diapausing female *Culex pipiens*, the expression of genes encoding ILP-1 and ILP-5 is downregulated. Silencing ILP-1 alone is sufficient to halt ovarian development, mimicking the diapause state. This effect can be reversed by applying JH, suggesting a link between insulin signaling and JH production [1, 7-10, 54-55].

FoxO levels are significantly elevated in the fat body of diapausing female *Culex pipiens* compared to their non-diapausing counterparts. When JH is administered to females destined for diapause, FoxO activity decreases, and fat accumulation is reduced. These findings further support a functional interaction between JH and FoxO that is critical for initiating and maintaining diapause [1, 7-10, 54-55].

In the mosquito *Culex pipiens*, FoxO knockdown in females programmed for diapause prevents the accumulation of lipid reserves essential for survival during this period. Using ChIP-sequencing to identify genes directly regulated by FoxO, researchers found that this transcription factor targets a broad set of genes involved in stress resistance, metabolism, lifespan extension, cell cycle control, growth regulation, and circadian rhythms in *Culex pipiens* [54-55].

Further evidence of FoxO's central role in diapause comes from knockdown experiments targeting three FoxO-regulated genes: glycogen synthase, an ATP-binding cassette transporter, and a low-density lipoprotein receptor chaperone. In the mosquito *Culex pipiens*, suppressing the activities of these three genes impaired glycogen and lipid synthesis, preventing proper energy storage and further confirming FoxO's importance in regulating diapause physiology. In the cotton bollworm (*Helicoverpa armigera*), high levels of the enzyme PRMT1 in the brain prevent FoxO phosphorylation. This leads to FoxO accumulation in brain cells during the pupal stage, thereby promoting entry into diapause [54-55].

These studies suggest that FoxO acts as a master regulator of the diapause state. Rather than individual genes acting in isolation, key transcription factors like FoxO may orchestrate multiple pathways to generate the complete diapause phenotype.

However, not all aspects of diapause are controlled solely by FoxO. In *C. pipiens*, FoxO influences both lipid storage and suppression of ovarian development. Similarly, FoxO regulates energy storage and stress responses during embryonic diapause in the locust *Locusta migratoria*. Other transcription factors also contribute: for example, the circadian transcription factor *Vrille* regulates egg development, while *Pdp1* influences fat accumulation. This indicates that multiple transcription factors act in both interconnected and independent ways to coordinate the full diapause program [54-55].

Integration of AKH, Corazonin, and Wnt Signaling in Insect Diapause Regulation

Adipokinetic hormones (AKHs) are a group of small neuropeptides composed of eight, or sometimes nine, amino acids. These hormones are produced by the corpora cardiaca and play a key role in mobilizing and releasing lipids, glycogen, and, in some cases, amino acids for use by the insect body. Based on their known functions, AKHs are also believed to contribute to diapause in insects [1, 56].

AKH binds to specific receptors on fat body cells—the insect equivalent of the liver and adipose tissue—activating intracellular signaling pathways that increase cyclic AMP (cAMP) and calcium ion (Ca^{2+}) concentrations. Elevated levels of these second messengers, in turn, activate lipase enzymes that break down triacylglycerol (TAG), releasing free fatty acids (FFA) that are used to generate ATP, the primary energy currency of the cell. This lipid mobilization process is integrated with other hormonal and signaling networks, including those involving juvenile hormone (JH), insulin, and the transcription factor FoxO, all of which are critical for regulating lipid storage and utilization [1, 56].

In the linden bug (*Pyrrhocoris apterus*), adult females in diapause release nearly twice as much lipid into the hemolymph following AKH injection compared to non-diapausing females. This indicates heightened sensitivity to AKH in the diapause state. Similarly, in fruit flies (*Drosophila melanogaster*), AKH mRNA levels increase more than fourfold during the first three weeks of diapause and remain approximately twofold higher throughout the rest of the dormancy period [1, 56].

The AKH family also includes corazonin, another neuropeptide that may participate in diapause regulation. Corazonin has diverse roles across insect species. It contributes to cuticle pigmentation, particularly in producing darker colors in locusts, a trait that may be linked to diapause in other insects. Corazonin is co-localized with the clock gene *period* in specific brain cells of the tobacco hornworm (*Manduca sexta*), indicating its potential role in circadian control. Destruction of these brain cells disrupts the diapause response. In the silkworm (*Bombyx mori*), corazonin is also involved in signaling the release of diapause hormone (DH) [1, 56].

During diapause induction, expression of genes encoding insulin-like peptides (ILPs) is downregulated, preventing the insulin receptor from inactivating FoxO. This allows FoxO to remain active, thereby promoting fat storage. During diapause maintenance, AKH production increases in response to elevated AMP-activated protein kinase (AMPK) activity. This stimulates the breakdown of TAG into diacylglycerol (DAG) via cAMP and Ca^{2+} signaling pathways [1, 54-56].

Recent studies on the cotton bollworm (*Helicoverpa armigera*) have shed light on the role of Wnt signaling in diapause. Under normal developmental conditions, the steroid hormone ecdysone activates the transcription factor *c-Myc* via the Wnt/β-catenin pathway. However, during diapause—when ecdysone levels are reduced—Wnt signaling becomes inactive in the pupal brain. Experimentally inhibiting the Wnt/β-catenin pathway produces a diapause-like state, suggesting that suppression of this signaling pathway is essential for diapause induction. Both transcriptomic analyses and functional experiments in *H. armigera* indicate that deactivation of Wnt signaling is a critical component of diapause entry [57].

Role of TGF-β and BMP Signaling Pathways in Hormonal Crosstalk and Diapause Regulation in Insects

The TGF-β (Transforming Growth Factor-β) signaling pathway plays a significant role in diapause regulation, particularly through its interaction with the juvenile hormone (JH) signaling pathway. The final step in JH biosynthesis involves converting a precursor molecule into the active hormone, a reaction catalyzed by juvenile hormone acid O-methyltransferase (JHAMT), which is encoded by the *jhamt* gene. The TGF-β family member

myoglianin suppresses *jhamt* expression, thereby reducing JH levels. This mechanism is associated with nymphal diapause in the cricket *Modicogryllus siamensis*, underscoring the intricate crosstalk among JH, insulin/target of rapamycin (TOR), and TGF-β signaling pathways in regulating diapause [1, 58].

In *Helicoverpa armigera*, the TGF-β signaling pathway negatively regulates the insulin signaling pathway to promote the diapause phenotype. Additionally, the BMP (Bone Morphogenetic Protein) signaling pathway is involved in diapause regulation in *H. armigera*. In diapause-destined pupae, both TGF-β and BMP signaling are downregulated in the brain, resulting in reduced expression of key downstream molecules such as phosphorylated Smad (p-Smad), POU domain transcription factors, and TFAM. These two signaling pathways appear to converge on the Smad1 transcription factor, which activates the POU promoter, leading to increased TFAM expression and alterations in metabolic regulation. When these pathways are inhibited, expression of downstream targets decreases and developmental progression is delayed. These findings indicate that downregulation of TGF-β and BMP signaling is integral to the diapause program, while reactivation of these pathways is essential for resuming post-diapause development, particularly by triggering ecdysone biosynthesis [1, 58].

Role of Histone Modifications and Chromatin Remodeli ng in Insect Diapause

Histone deacetylation is generally associated with gene silencing. In the flesh fly *Sarcophaga bullata*, pupal diapause is accompanied by an approximately 75% reduction in acetylated histone H3 levels, indicating widespread suppression of gene transcription. Interestingly, this reduction occurs alongside decreased activity of histone deacetylases (HDACs) and downregulation of several critical genes involved in chromatin remodeling. These include hdac3, hdac6, sirt1, sirt2, and the histone acetyltransferase (HAT) gene *gnc5*, all of which are suppressed during diapause [1, 59].

These findings suggest that global histone deacetylation may not primarily occur during diapause itself, but rather during the photosensitive larval stage that precedes diapause. This is further supported by increased expression of genes encoding HDACs and HATs during that earlier stage, implying a preparatory role for chromatin remodeling in establishing the diapause state [1, 59].

Epigenetic and Post-Transcriptional Mechanisms Underlying Insect Diapause

The Polycomb Repressive Complex 2 (PRC2) plays a key role in controlling pupal diapause in the cotton bollworm, *Helicoverpa armigera*. Under long-day conditions, which inhibit diapause, expression of the Extra sex combs (Esc) gene, which encodes a component of PRC2, is elevated. This leads to increased levels of H3K27me3, a repressive histone mark. Interestingly, in this context, the epigenetic mark H3K27me3 does not repress—but rather activates—the expression of the *ptth* gene, which promotes development and terminates diapause in *Helicoverpa armigera*. When *esc* is silenced by RNA interference (RNAi) or PRC2 is chemically inhibited, H3K27me3 levels and PTTH production drop significantly, resulting in developmental arrest even in pupae that would normally continue developing [1, 59].

MicroRNA-Mediated Regulation of Diapause Across Insect Species

MicroRNAs (miRNAs) also contribute to diapause regulation. The expression levels of two miRNAs, let-7 and miR-100, decrease during diapause in both *Sarcophaga bullata* pupae and *Galeruca daurica* adults. Let-7 is known for its role in developmental timing, while miR-100 regulates cell cycle progression [59, 60].

In the mosquito *Culex pipiens*, which undergoes adult diapause, miRNA profiles shift between the pre-diapause and diapause stages, resembling the patterns observed in *S. bullata*. Specifically, the expression levels of miR-13b-3p, miR-275-3p, and miR-305-5p decrease during diapause in both species [59, 60].

In the silkworm *Bombyx mori*, miR-2761 is significantly more abundant—about fivefold higher—in diapausing embryos, and its expression sharply decreases after diapause ends. This miRNA inhibits the sdh (*sorbitol dehydrogenase*) gene, which catalyzes the conversion of sorbitol to glycogen—a process previously linked to diapause termination. By repressing *sdh*, miR-2761 helps maintain high sorbitol levels, ensuring the embryo remains in diapause [59, 60].

In *Helicoverpa zea*, pupal diapause can be terminated by injecting ecdysone, diapause hormone, or its analogs. These treatments consistently lead to downregulation of miR-277, a miRNA associated with insulin/FoxO signaling and metabolic regulation. This suggests that miR-277 plays a role in reactivating metabolism at diapause termination [59, 60].

Concluding Remarks

Diapause in insects is a multifaceted, genetically regulated developmental arrest that enables survival under adverse environmental conditions [7-11]. Recent advances in molecular biology, endocrinology, epigenetics, and transcriptomics have significantly enhanced our understanding of the intricate regulatory networks governing this adaptive process. This review synthesizes current knowledge across diverse yet interconnected domains—from metabolic suppression, nutrient storage, and stress tolerance to the orchestration of neuroendocrine, hormonal, and circadian pathways that mediate the onset, maintenance, and termination of insect diapause, a highly coordinated state of molecular reprogramming.

Our current understanding highlights the central roles of juvenile hormone (JH), ecdysteroids, insulin/FoxO signaling, and circadian clock genes in regulating the onset, maintenance, and termination of diapause across diverse insect taxa.

The roles of key central regulators—FoxO, JH, ecdysteroids, and prothoracicotropic hormone (PTTH)—in insect diapause are discussed alongside major signaling cascades, including the insulin/IGF, TGF-β/BMP, Wnt, and AKH/Corazonin pathways. These hormonal circuits converge with circadian and photoperiodic inputs to establish a robust temporal framework for diapause regulation. Moreover, cell cycle arrest, histone modifications, chromatin remodeling, and post-transcriptional gene regulation via non-coding RNAs add further depth to the diapause program, ensuring precise

modulation of gene expression in a stage-, tissue-, and condition-specific manner.

Species-specific studies, such as those in *Bombyx mori*, continue to provide valuable mechanistic insights into diapause regulation, highlighting both the evolutionarily conserved and diversified nature of diapause strategies across insect taxa. Despite these advances, critical gaps remain—particularly in decoding the epigenetic memory of diapause, the full scope of RNA-mediated regulation, and the integration of environmental cues with gene regulatory networks.

The molecular triggers for diapause termination, the roles of epigenetic modifications, and the mechanisms by which environmental inputs are processed at the neuroendocrine level warrant further investigation. Future research employing CRISPR/Cas9, single-cell transcriptomics, and multi-omics approaches will be instrumental in unraveling the remaining molecular and regulatory complexities of insect diapause—an anticipatory survival strategy.

A deeper understanding of diapause at the molecular level not only enriches our knowledge of insect developmental biology and evolution but also offers promising applications in pest management, pollinator conservation, and insect biotechnology.

In sum, diapause in insects represents a powerful model system for studying dynamic gene–environment interactions. Continued exploration into its molecular regulation holds significant promise for both fundamental biological insight and practical innovation.

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