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REVIEW

Diapause among the flesh flies (Diptera: Sarcophagidae)*

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Abstract. The rich diversity of information focusing on pupal diapause in the sarcophagids makes this fly family among the best-understood diapause models. This review summarizes the occurrence of pupal diapause in flesh flies from broad geographic regions of the world, as well as the apparent absence of diapause in select regions. The environmental cues used for programming diapause are discussed, as well as the requirements for breaking diapause. This taxon has been used for experiments ranging from the ecological to the molecular and offers a comprehensive overview of the diapause phenotype. A wide range of diapause attributes define the diapause phenotype of flesh flies, offering insights into such features as clock mechanisms, signaling pathways, maternal regulation, energy utilization, cell cycle regulation, metabolic depression, cyclic metabolic activity, cold tolerance, water balance, and other attributes, generating a diapause profile that offers an attractive comparison for diapause in other insect species as well as with other forms of animal dormancy.

INTRODUCTION

I first encountered diapause in the Sarcophagidae as a graduate student at the University of Illinois in the late 1960s. Professor Gottfried Fraenkel had recently returned to Illinois with pupae of *Sarcophaga argyrostoma* he had obtained from the University of Paris (Quai Saint-Bernard). Some flies emerged immediately, but others failed to do so and were locked into a developmental arrest as pupae. He and Catherine Hsiao documented this pupal diapause in *Sarcophaga argyrostoma* and *S. bullata*, along with descriptions of the environmental conditions needed to induce the diapause and certain features of the diapause morphology (Fraenkel & Hsiao, 1968a, b). At that time, diapause was well known in pupae of Lepidoptera, but it was not widely acknowledged in Diptera. (There were, however, older papers by Roubaud (1922) reporting diapause in *Sarcophaga* and by House (1967) that mentioned pupal diapause in *Agria affinis*, a flesh fly parasite of the spruce budworm). Fraenkel had pioneered an incredibly diverse range of topics in insect physiology and behavior, but he had not previously investigated diapause. He persuaded a young faculty member, Professor Judith Willis, who had co-authored a study on larval diapause in the parasitoid *Nasonia vitripennis* (Schneiderman & Horwitz 1958), to help guide me into the diapause literature, and together we started probing metabolic rates in diapausing flesh flies (Denlinger et al., 1972). I was hooked. Those initial experiments fostered my lifelong interest in diapause, especially pupal diapause in flesh flies. Since that time, flesh flies have garnered considerable attention as a diapause model, and my goal here is to briefly summarize the current scope of our knowledge, with the hope of providing a springboard for new investigators interested in exploring the many remaining questions related to diapause in this interesting family.

Consistently, at temperate latitudes, third instar larvae complete feeding, leave the food source, burrow into the substrate and pupariate. In response to appropriate environmental cues received early in larval life, the pupa inside the puparium enters a facultative diapause. Though some insect species enter summer diapause, and some enter diapause repeatedly or may enter a prolonged diapause lasting more than a year (Denlinger, 2022), no such features have been noted in the sarcophagids. Diapause is exclusively entered in the pupal stage and serves to bridge the winter season or tropical cool seasons, as discussed below. With over 3000 described species in the family Sarcophagidae worldwide (Pape, 1996), there will likely be some species that do not conform to the diapause observations documented here, but the striking observation thus far is how uniform the pupal diapause response appears to be.

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The sarcophagids are unusual in that they are ovoviviparous, meaning that the eggs, after fertilization, are retained within the female's reproductive tract until completion of embryogenesis (Denlinger, 1971). The mother then deposits active first instar larvae that are immediately able to start feeding. Unlike tsetse flies, in which the mother provides nutriment for her progeny, female sarcophagids provide no nutriment but simply retain the embryos within the uterus during the 4–6 days required to complete embryogenesis. This live-birth strategy enables larvae to quickly exploit a food resource and would appear to offer a competitive advantage to larvae feeding on a carcass resource that may be highly contested by other scavengers. Many sarcophagids are parasitic (Aldrich, 1916), and, for such species, being deposited as an active larva enables rapid penetration of the host's cuticle, without the delay of waiting for egg hatch.

The reliance of many flesh fly species on carrion and animal excreta as food makes them important contributors to the food chain as decomposers (Szpila et al., 2015), but they can also infest wounds of living animals, resulting in myiasis, an issue especially noteworthy among goats and sheep (and occasionally humans) in southern Europe, around the Mediterranean, and in Australia and Japan (Solar Cruz et al., 1996; Ternovoy, 1978; Monzu, 1979; Miura et al., 2005). As feeders on decaying flesh, they can also be important forensic indicator species providing insights on time of death (Shang et al., 2019). A number of sarcophagids are parasitic on large-bodied beetles, grasshoppers and caterpillars, as well as snails and other invertebrates. *A. affinis*, for example, is a significant parasite of the spruce budworm across its range in the northern Rocky Mountains in the USA and Canada (House, 1967), and *S. villeneuveana*, a native of southern Europe, is a major parasite of conical snails, and as such has been introduced into Australia to control *Cochlicella acuta*, a snail that was itself introduced to Australia from the Mediterranean region and became established as a pest of pastures and grain crops (Muirhead, 2021).

The ease of rearing flesh flies in the laboratory (Denlinger, 1972a), their large size and short generation time contribute to their attractiveness as a research model. In addition to their usefulness as a model for diapause, the flies have proven valuable in insect endocrinology by revealing numerous pupariation factors (Žďárek, 1985) as well as a rich collection of other neuropeptides, most with functions unknown (Verleyen et al., 2004). The sarcophagids have featured prominently in the literature on metamorphosis behavior (e.g. Denlinger & Žďárek, 1994), neurobiology (e.g. Wasserman & Itagaki, 2003), reproduction (e.g. Briers & de Loof, 1980), and community ecology (e.g. Hanski, 1981). The presence of giant polytene chromosomes in male foot pads offered a valuable tool in early experiments probing chromosome puffing (Whitten, 1969). Molecular tools are now available for sarcophagids from an EST project on *S. crassipalpis* (Hahn et al., 2009) and the genome sequencing of *S. bullata* (Martinson et al., 2019). In addition, *Nasonia vitripennis,* a parasitoid that favors the sarcophagids as a host (Rivers & Denlinger, 1995), has been sequenced (Werren et al., 2010), thus allowing detailed monitoring of molecular interactions elicited in this host-parasitoid relationship (Danneels et al., 2013). A catalog on the Sarcophagidae of the world (Pape, 1996), augmented by recent, detailed studies on the systematics and phylogeny of the genus *Sarcophaga* (Giroux & Wheeler, 2009; Buenaventura et al., 2017) provide rich and critical foundations for understanding taxonomic features of this

Table 1. Representative records for diapause or lack thereof in species of Sarcophagidae, along with collection localities. When diapause has been noted, it has consistently been in the pupal stage.

Species	Latitude	Country	Diapause present	Reference
Agria affinis	50°N	Canada	yes	House, 1967
Euboettcheria trejosi	9°N	Panama	no	Denlinger & Shukla, 1984
Pattonella intermutans	9°N	Panama	no	Denlinger & Shukla, 1984
Peckia abnormis	9°N	Panama	no	Tanaka et al., 1987
P. chrysostoma	9°N	Panama	no	Tanaka et al., 1987
Poecilometopa spilogaster	1°S	Kenya	yes	Denlinger, 1979
P. punctipennis	1°S	Kenya	yes	Denlinger, 1979
Sarcodexia sternodontis	9°N	Panama	no	Tanaka et al., 1987
Sarcophaga argyrostoma	49°N	France	yes	Fraenkel & Hsiao, 1968a
S. bullata	40°N	USA	yes	Fraenkel & Hsiao, 1968a
S. crassipalpis	40°N	USA	yes	Denlinger, 1972a
S. exuberans	1°S	Kenya	yes	Denlinger, 1979
S. formosensis	22°N	Hong Kong	yes	Kurahashi & Ohtaki, 1989
S. haemorrhoidalis	4°S	Kenya	yes	Denlinger, 1979
S. inzi	1°S	Kenya	yes	Denlinger, 1979
S. monospila	9°N	Ethiopia	yes	Denlinger, 1979
S. nathani	36°N	Pakistan	yes	Kurahashi & Ohtaki, 1989
S. par	9°S	Tanzania	yes	Denlinger, 1970
S. peregrina	7°S	New Guinea	yes	Kurahashi & Ohtaki, 1989
S. ruficornis	1°S	Brazil	yes	Denlinger, 1979
S. septentrionalis	43°N	Russia	yes	Vinogradova, 1976
S. similis	35°N	Japan	yes	Goto, 2009
S. villeneuveana	35°S	Australia	yes	Muirhead & Perry, 2021
Tricholioproctia impatiens	38°S	Australia	yes	Roberts & Warren, 1975
Wohlfahrtia magnifica	37°N	Spain	yes	Soler Cruz et al., 1996

group of flies. The breadth of information available on the flesh flies enhances their value as a model for probing life history traits that are central to understanding seasonality and the mechanisms engaged to invoke the diapause state.

1. Universal reliance on the pupal stage for diapause

In some insect families, and even within certain genera, diapause occurs in multiple developmental stages (Denlinger, 2022), but that is not the case in the family Sarcophagidae. All reported diapauses in this family occur in the pupal stage (Table 1). As indicated in the table, this is true regardless of geographic origin. Flesh flies in diverse genera from North America, Europe, Asia, and Africa all rely on a pupal diapause. This is also true for *S. ruficornis,* a species sampled from Brazil, but I should note that this species is cosmopolitan and is thought to have been introduced into South America from the Old World. Table 1 is not an exhaustive list of flesh fly species having a pupal diapause. For example, additional species listed by Vinogradova (1976) refer to pupal diapause in several sarcophagid species discussed in papers I have not been able to access, e.g. *Sarcophaga melanura, S. semenovi* and *Ravinia striata.*

The pupal stage, with its protective puparium and inherent low metabolic rate, would appear to be ideal for diapause, but obviously other species execute successful diapauses as embryos, larvae or adults, so the pupal stage is not inherently the best stage for diapause in all species. Pupariation sites are usually underground, thus the pupa within the puparium is also naturally buffered from the full onslaught of winter temperatures. On the downside, pupae in underground sites must be defended against fungi and other soil microbes, and the moist soil environment is subject to freezing, making pupae vulnerable to inoculative freezing from surrounding ice crystals. And, the immobility of the pupal stages means that it is especially important for larvae to select a site that will remain favorable for the many months of diapause. It is thus interesting to note that the wandering phase of third instar larvae programmed for pupal diapause is considerably longer than the comparable phase for larvae not destined for diapause (Denlinger, 1972a), a feature that perhaps allows larvae to more thoroughly evaluate a potential pupariation site.

2. When diapause is absent

Interestingly, there are species in which diapause appears to be absent. This is best documented in five species from Panama: *Euboettcheria trejosi, Pattonella intermutans, Peckia abnormis, P. chrysostoma* and *Sarcodexia sternodontis* (Table 1). These species have rigorously been evaluated for diapause (Denlinger & Shukla, 1984; Tanaka et al., 1987), and no diapause was observed under a range of photoperiods and temperatures that elicit diapause in flesh flies from temperate areas or tropical Africa. Is diapause perhaps not present in lineages from the New World tropics? It will be interesting to see if this remains true as more South and Central American species are examined. The presence of diapause in Brazilian populations of *S. ruficornis* does not refute this idea since this cosmopolitan species appears to have originated in the Old World.

Correlated with a lack of diapause in the flies from Panama is a marked increase in variability and duration of the post-feeding, wandering phase of the third larval instar and an increase in adult longevity (Denlinger & Shukla, 1984). For example, median duration for the post-feeding wandering phase is 6–7 days for two Panamanian flies that lack diapause (*E. trejosi* and *P. intermutans*), compared to 2 days for tropical flies having a diapause (*Sarcophaga ruficornis* and *S. par*). Median adult life span is 12 to 37 days longer for the two Panamanian flies and may be up to 65 days longer than in flies with a diapause. The longer adult life does not result in higher reproductive output than noted for temperate species. The same total number of progeny are produced, but they are simply produced over a longer period of time. These attributes suggest an alternative to diapause. The differences between the Panama flies and all the others suggest a scenario where species with a pupal diapause quickly move through other life stages and invest all their risk in a pupal diapause, whereas those lacking diapause spread risk over their entire life cycle, resulting in variable and longer larval and adult life spans.

Certain geographic populations of *S. peregrina* (from Papua New Guinea, 6°S) are reported not to have a diapause (Kurahashi & Ohtaki, 1977). A lack of diapause is also reported for a number of other species and populations from the Asian subtropical and tropical Oceanic islands, including *S. karnyi, S. koimani, S. timorensis* and *S. invaria* (Kurahashi & Ohtaki, 1989), but we cannot say for certain whether diapause exists in these population. What has been shown is that these populations and species are not responsive to photoperiod at 20 or 28°C. Such conditions would not detect the sort of diapause noted in tropical African flies. The African flies also lack a photoperiodic response, yet they have a diapause induced by low temperature (Denlinger, 1974, 1979).

3. Attributes of diapause in sarcophagids

A. Prolongation of the post-feeding wandering phase prior to diapause

In many insect species, the prediapause developmental rate is influenced by the diapause program, resulting in either an accelerated or slower rate of development prior to diapause (Denlinger, 2022). A slowing of development may allow a diapause-destined insect to accumulate more reserves, but a more rapid rate of development may be needed to reach the diapausing stage before the advent of winter. In flesh flies, the diapause program does not influence duration of larval feeding, but the post-feeding, wandering phase of the third instar is longer in those destined for pupal diapause (Denlinger, 1972a). For example, larvae of *S. bullata* not destined for diapause pupariate 1–2 days after leaving the food, whereas those destined for diapause may wander for up to two weeks before pupariating. The delay is not always as pronounced in other flesh fly species as it is in *S. bullata*, but consistently some sort of delay is noted. Since this retardation of development occurs after

feeding has ceased, it does not contribute to the accumulation of more energy reserves. It may facilitate finding a more secure site for overwintering, but the ecological significance of this life history trait remains obscure.

B. Developmental arrest

In some diapausing insects, slow but distinct developmental progression can be noted during diapause, but in the pupal diapause of sarcophagids, there are not obvious signs of developmental progression during diapause. Development is halted in the phanerocephalic pupal stage, shortly after head eversion but before the antennal discs begin their migration from a central to a more lateral position in the head (Fraenkel & Hsiao, 1968b). The combined ganglion present in larvae has not yet begun to separate into ganglia of the head and thorax. The fat body has broken down into single cells but has not degenerated further. These characteristics are all identical to those observed in nondiapausing individuals approximately 40 h after pupariation at 29°C. Pupae remain developmentally locked in arrest at that stage until diapause is broken. The first visual indicator of diapause termination is migration of the antennal discs and their transformation from a circular shape to an elongated form. A boost in metabolic rate can be detected a few days prior to antennal disc migration (Denlinger et al., 1972), and, of course, certain molecular indicators of development also precede, by a few days, the physical migration of the discs.

C. Arrest of the cell cycle

During pupal diapause in *S. crassipalpis* brain cells are arrested at the G0/G1 phase (Tammariello & Denlinger, 1998), an arrest that correlates with downregulation of the transcript *proliferating cell nuclear antigen* (*pcna*), a transcript that encodes a protein involved in the G1/S phase transition (Flannagan et al., 1998). The shutdown in expression of *pcna* thus emerges as a key regulatory site for arresting the cell cycle during flesh fly diapause, but it is likely not the only site targeted. Another key cell cycle regulator may be *cyclin-dependent kinase 1* (*cdk1*), as suggested by a phosophoproteomic analysis of the brain of *S. crassipalpis* (Pavlides et al., 2011). As more information accumulates, it is evident that different phases of the cell cycle may be targeted for shutting down the cell cycle in different insects, but consistently the cell cycle is targeted as a key component of diapause, regardless of species (Denlinger, 2022).

D. Reduced protein synthesis

Pulse labeling experiments with *S. crassipalpis* reveal that the overall rate of protein synthesis is greatly reduced during diapause, as evidenced in whole body samples (Joplin & Denlinger, 1989) as well as brains (Joplin et al., 1990). Rates of synthesis are approximately eight-fold higher in nondapausing states. Although the rate of protein synthesis remains consistently low throughout diapause, there is some variation, correlating with the cycles of oxygen consumption discussed below. A higher rate of synthesis is noted during the peaks in the oxygen cycle than during the troughs.

E. Metabolic depression and infradian patterns of metabolism

Metabolic depression is especially impressive for all pupal diapauses, and this holds true for the sarcophagids as well. In these flies, the nadir of the U-shaped curve of metabolic rate characteristic of the metamorphic transition from larva to adult in nondiapausing individuals is approximately 150 ul/g/h at 25°C. In diapausing pupae the metabolic rate drops far lower, to a mean value of 10–20 ul/g/h, a rate less than 10% of the lowest nondiapausing rate (Denlinger et al., 1972). Metabolic rates at pupariation or adult eclosion (> 800 ul/g/h) are 80× higher than during diapause. The metabolic depression during diapause thus offers considerable economy in the utilization of energy reserves, a key feature for bridging the many months of diapause.

But, the feature that is most striking about metabolic depression in flesh flies is the fact that the metabolic rate is not constant during diapause. The metabolic rate regularly cycles between days with no or very little detectable oxygen consumption to days of high consumption with peaks of 50–80 ul/g/h (Denlinger et al., 1972). Each peak, which starts rather abruptly but declines gradually, lasts approximately 34 h at 24–27°C (Sláma & Denlinger, 1992). The interpeak periods of low respiration last 4–5 days at 25°C, but extends to nearly 10 days at 18°C (Denlinger et al., 1972). Interestingly, these infradian cycles are not of constant duration: cycles are closer together in early diapause, become further apart in mid-diapause, and then again become closer together toward the end of diapause. In many ways these cycles are reminiscent of the periodic cycles of arousal described in hibernating ground squirrels (Pengelley & Fisher, 1961). Juvenile hormone (JH) signaling (Denlinger et al., 1984) as well as ROS and hypoxia signaling pathways (Chen et al., 2021) contribute to the coordination of these cycles. Like mammalian hibernators, these cycles in flesh flies represent a switch from anaerobic metabolism during the depression phase of the cycle to aerobic metabolism during the periods of arousal (Chen et al., 2021), underscoring an interesting parallel between mammalian hibernation and insect diapause.

These metabolic cycles should not be confused with periods of cyclic carbon dioxide release reported for diapausing pupae of Lepidoptera (Schneiderman & Williams, 1953). In diapausing Lepidoptera, the spiracular valves periodically open, releasing a burst of carbon dioxide, but in those cases, the rate of oxygen consumption remains constant throughout, thus the flesh fly cycles differ fundamentally from the well-known cyclic release of carbon dioxide reported for diapausing Lepidoptera.

F. Energy management

Since pupae lack the ability to feed during diapause, the reserves needed to survive 7–9 months of diapause and then to complete adult differentiation must be acquired prior to diapause entry. An early report suggested that flesh

flies destined for diapause accumulate more lipid reserves than those not destined for diapause (Adedokun & Denlinger, 1985), as noted in numerous other insect species (Denlinger, 2022), but that appears to be a spurious result because more recent experiments show no substantial differences in weight or lipid accumulation between diapause and nondiapause destined pupae of *S. crassipalpis* (Hahn & Denlinger, unpubl. results). What is clear however, is that the diapausing pupae rely on lipid reserves during the first half of diapause and then switch midway to nonlipid reserves (Adedokun & Denlinger, 1985). When JH is applied to diapausing pupae, the metabolic rate is elevated, resulting in a much shorter diapause (Denlinger et al., 1984). This observation and others suggest that diapausing pupae have the ability to monitor their energy reserves and break diapause when those reserves become dangerously low.

A metabolomic comparison of diapausing and nondiapausing pupae of *S. crassipalpis* reveals that diapause is associated with increased levels of metabolites involved in glycolysis and decreased levels of components of the TCA cycle (Michaud & Denlinger, 2007), a result that is also reflected in a transcriptome analysis (Ragland et al., 2010). As in many other diapauses, there is a major shift from aerobic to anaerobic metabolism during diapause in flesh flies.

Elevation of the transcript encoding phosphoenolpyruvate carboxykinase (Pepck) is especially noteworthy in the diapause of *S. crassipalpis* (Ragland et al., 2010), and other species as well (Denlinger, 2022). This anaerobic metabolic enzyme catalyzes oxaloacetate into CO_2 and phosphoenol pyruvate, a metabolite that can be used in gluconeogenesis, a result underscoring the dominance of anaerobic pathways during diapause. There are two isoforms of PEPCK, a cytosolic (PEPCK-C) and mitochondrial (PEPCK-M) form; both are elevated during pupal diapause in *S. crassipalpis* (Spacht et al., 2018).

G. Heartbeat

As the metabolic rate drops during diapause in *S. crassipalpis*, the heartbeat rate also declines, and the heart may stop beating for up to 30 min (Sláma & Denlinger, 2013). Periods of cardiac rest are interrupted by short bouts of fast heartbeats. All pulsations are anterograde (forward directed) during diapause, but periodic retrograde pulsations that push the hemolymph backwards are initiated within the abdomen when diapause is terminated.

H. Enhanced cold tolerance and other stress responses

Cold hardiness is the stress response most extensively examined in diapausing sarcophagid pupae. Unlike some insects, flesh fly pupae cannot tolerate freezing, but by lowering their supercooling point (SCP) to approximately –23°C, pupae of *S. crassipalpis* can avoid freezing at temperatures down to approximately the SCP (Lee & Denlinger, 1985). Though nondiapausing pupae at a stage equivalent to that of diapause also have an SCP of –23°C, they cannot tolerate temperatures below –17°C. Cold tolerance progressively increases during the first 10 days of diapause, remains elevated throughout diapause and then is quickly lost at diapause termination, a dynamic that coincides with the elevation and subsequent drop in titers of glycerol, a polyol that appears to be the major cryoprotectant used by these flies (Lee et al., 1987). In addition to glycerol, other conspicuous metabolites elevated in association with diapause include glucose, alanine and pyruvate (Michaud & Denlinger, 2007). Several heat shock proteins (Hsps) are upregulated in association with diapause, and their knockdown by RNAi reduces cold tolerance, indicating an important role for these stress proteins in protecting diapausing pupae from cold injury (Rinehart et al., 2007).

In some insect species, cold tolerance is simply coincidental with diapause, i.e. it normally coincides in time with diapause but is prompted by a distinct set of environmental cues, usually the onset of low temperatures. In other cases diapause and cold tolerance are linked, i.e. cold tolerance is evoked by the entry into diapause (Denlinger, 1991). In the sarcophagids, the two are linked. Entry into diapause elicits enhanced cold tolerance, suggesting that cold tolerance is a component of the diapause program in these flies (Adedokun & Denlinger, 1984).

Other stress responses are also in evidence during flesh fly diapause. The immune signaling genes *cactus* and *dorsal* (an activator of the antimicrobial peptide diptericin), along with *defensin,* an antimicrobial peptide, are elevated during pupal diapause in *S. crassipalpis* (Ragland et al., 2010). While these three transcripts are all elevated as a function of diapause, other immune-related genes such as *sarcotoxin II* are elevated during diapause only in response to an immune challenge (Rinehart et al., 2003).

Interestingly, the antioxidant enzymes commonly associated with oxidative stress such as catalase, glutathione peroxidase, or superoxide dismutase are not elevated during pupal diapause in *S. crassipalpis* as they are in some other diapausing species, but levels of ferritin, an oxygen scavenger, and other metalloproteins are high (Ragland et al., 2010), perhaps providing an alternative form of protection against oxidative stress for these flies during diapause. Elevated selenoproteins and metalloproteins levels (Rinehart et al., 2010) may be important not only in detoxification but also in the immune response.

Diapausing pupae are also more tolerant of anoxia. While nondiapausing pupae of *S. crassipalpis* survive only one day of anoxia, diapausing pupae can survive anoxia for up to six days (Kukal et al., 1991). This feature may be especially important for flesh fly pupae because they overwinter in an oxygen-limited underground site subjected to flooding and freezing.

I. Maintaining water balance

A diapausing pupa is, of course, unable to acquire new water resources by drinking, thus maintenance of water balance is almost exclusively dependent on restricting water loss. This is not a trivial challenge considering the many months spent by the flies in pupal diapause. Net transpiration rates are considerably lower for diapausing pupae of *S. crassipalpis* (Yoder & Denlinger, 1991), partially due to the lower rates of metabolism but also due to impressive properties of the puparium. Additional hydrocarbons line

the inner surface of the puparia of diapausing individuals, thus providing a bolstered layer of waterproofing (Yoder et al., 1992). The same types of hydrocarbons are present on the puparial surfaces of both diapausing and nondiapausing pupae, but the puparia of diapausing flies contain twice as many hydrocarbons. The distinction between the two types of pupae is also evident when the critical transition temperature (CTT) is measured (Yoder & Denlinger, 1991). The CTT represents the inflection point on a temperature curve depicting water loss rates, and this value is 9°C higher for diapausing flesh fly pupae. Although the basis for the CTT remains controversial, a conservative interpretation is that it reflects the quantity and/or quality of the hydrocarbons present and is indicative of water loss properties. Metabolic water appears to contribute little to the water needs during diapause, but some water may be actively acquired through a poorly understood water vapor absorption mechanism (Yoder & Denlinger, 1991).

4. Reading environmental cues for diapause induction in temperate latitudes

Like most insect species living in temperate latitudes, the sarcophagids rely on photoperiod for the programming of diapause. Short daylengths are diapause-inducing, while diapause is absent under long daylengths. The transition between diapause-inductive photoperiods and those not inducing diapause, defined as the critical daylength (CDL), occurs over a remarkably narrow photoperiodic range. For populations of *S. bullata* from 40°N in North America, the CDL is 13.5 h of light per day; at a light : dark cyle of 13L : 11D nearly all individuals enter diapause and at 14L : 10D diapause is nearly absent (Denlinger, 1972a). Similar steep photoperiodic curves are also noted in *S. crassipalpis* (Gnagey & Denlinger, 1984), *S. similis* (Yamaguchi & Goto, 2019; Moribayashi et al., 2021) and *S. peregrina* (Moribayashi et al., 2021). CDLs have been calculated for different geographic populations of *S. similis* (Yamaguchi & Goto, 2019) and *S. peregrina* (Moribayashi et al., 2021), and as in other insect species, the CDLs of both species shift with latitude: fly populations at more northerly latitudes have longer CDLs. Yamaguchi & Goto (2019), in a particularly insightful paper, propose that selection is acting on timing of the photoinducible phase within the daily 24 h light : dark cycle to generate the latitudinal cline of CDL noted for populations of *S. similis.*

The photosensitive period used to program pupal diapause can be quite brief in the sarcophagids. In *S. crassipalpis*, the final two days of embryonic development and the first two days of larval development are sufficient to induce pupal diapause (Denlinger, 1971). Though the embryos are being held within the mother's uterus, the photoperiodic cues are transmitted directly through the abdomen of the mother to the embryos within. Similar embryonic sensitivity is noted as well in *S. peregrina* (Kurahashi & Ohtaki, 1979), *S. similis* and *S. septentrionalis* (Vinogradova, 1976), although the duration of sensitivity during larval life may vary with species. Experiments with *S. argyrostoma* show that a lengthy period of larval exposure to short days can induce diapause, even if the period of embryonic sensitivity is missed (Saunders, 1971). Manipulation of the duration of larval exposure to short daylengths has resulted in an attractive model suggesting the presence of a Required Day Number (RDN), a specific number of short days needed to program diapause (Saunders, 1971). For *S. argyrostoma*, the RDN is 14 days, thus offering a model that explains why high temperature that results in completion of larval development in a much shorter time will result in nondiapause, while low temperatures that delay development require periods of larval development exceeding 14 days, resulting in pupal diapause.

The extensive experimentation on the role of circadian rhythms in the programming of diapause is beyond the scope of this review, but experiments with *S. argyrostoma* (see comprehensive reviews by Saunders, 2002, 2020) and more recently *S. similis* (Yamaguchi & Goto, 2019) have featured prominently in these advances. The programming of diapause in flesh flies is consistent with an "external coincidence model", implicating an endogenous circadian oscillator with a photoinducible phase occurring late in the subjective night.

Photosensitivity is restricted to the blue region of the spectrum (less than 540 nm), suggesting involvement of a blue-light mediated receptor in mediating the photoperiodic response of diapause in flesh flies (Gnagey & Denlinger, 1984), and the canonical clock genes are most certainly involved. In *S. crassipalpis*, short days and long days generate distinct expression patterns for *period, timeless, cycle* and *cryptochrome* during the photosensitive period (Goto & Denlinger, 2002; Koštál et al., 2009). In *S. bullata,* a nondiapausing variant that shows an arrhythmic adult eclosion pattern expresses both *period* and *timeless* at considerably higher levels than seen in the wild type flies (Goto et al., 2006). The length of the *period* gene in different variants of *S. bullata* correlates with the incidence of pupal diapause (Han & Denlinger, 2009), a result that again points to a critical role for the clock genes in transducing the environmental signal of short daylength for the programming of diapause. But, what is still lacking are clear results from gene knockdown experiments. During diapause itself, the clock most likely ceases to cycle (Short et al., 2016).

Consistently, the effect of daylength on the diapause program is augmented by temperature, with lower temperatures generating higher diapause incidences. For example, if larvae of *S. crassipalpis* are reared under short daylengths at 28°C, diapause incidence is 57%, at 25°C diapause incidence is 86%, and at 17°C the incidence is 100% (Denlinger, 1972a). Very few enter diapause if low temperatures are not coupled with short days. In some insect species, temperature affects critical daylength, while it has no such influence in others (Denlinger, 2022). Although this aspect has not been examined extensively in sarcophagids, results for *S. argyrostoma* show no difference in critical daylength for flies reared at 15 and 18°C (Saunders, 1971).

5. What programs diapause in the tropics?

As shown in Table 1, pupal diapause is present in a number of sarcophagids from the Old World tropics but not from the New World tropics, with the exception of a

cosmopolitan species, *S. ruficornis*, that presumably was introduced to the New World by human activity. Numerous species near the equator and within 10° North and South of the equator in East Africa have a pupal diapause showing the same physiological attributes as their temperate latitude relatives (Denlinger, 1974, 1979). But, rather than being programmed by photoperiod, diapause in these African species is programmed by the low daytime temperatures that prevail during July and August. It is not immediately obvious what is driving selection for diapause in these tropical flies. Certainly temperatures are not so low as to prohibit development. Possibly they are simply using this abiotic feature of the East African seasons to periodically initiate a halt in development to escape other biotic factors in the environment. Diapause in these African flies is not long lasting and can easily be broken by exposure to a few days of high temperature.

6. Diapause incidence increases with mother's age

The incidence of pupal diapause in the female's progeny increases as the female ages (Rockey & Denlinger, 1986). Under conditions that result in 37% pupal diapause incidence in the first brood produced by *S. bullata* females, the diapause incidence progressively increases in subsequent broods. And, in females exhibiting the maternal effect discussed below, diapause is absent in the first brood, but by the third brood, produced 26 days later, the incidence of diapause rises to 24%. It remains unknown how age exerts this conspicuous effect on the production of diapausing progeny.

7. Water content of larval diet influences diapause incidence

In addition to photoperiod, temperature, and age of the mother, water content of the larva's food can also impact the diapause decision of the pupae. In both temperate latitude sarcophagids (Denlinger, 1972a) as well as in species from tropical Africa (Denlinger, 1979), adding 10% water to the larval diet boosts the incidence of pupal diapause approximately 10%, and conversely, reducing the water content by 10% for larvae of the African flies results in an 18% drop in the incidence of diapause.

8. Males enter diapause earlier than females

Males have a lower threshold for diapause, resulting in an earlier seasonal entry into diapause by males than females, as documented for *S. crassipalpis* (Denlinger, 1972a), *S. bullata* (Denlinger, 1972b) and *S. similis* (Yamaguchi & Goto, 2019). In *S. similis,* the sex distinction in diapause timing is more pronounced in more southern populations. Field observations in Osaka, Japan also confirm that males of *S. similis* enter diapause approximately two weeks earlier than females (Mukai et al., 2021).The fact that males are less sensitive than females to light exposure during the photoinductive period late in the scotophase also suggests that the timing mechanisms operate differently in the two sexes (Yamaguchi & Goto, 2019).

This sex difference reflects different costs of diapause in males and females. While a long diapause appears to have no costs for males, female fitness, measured as post-diapause egg production and fertility, declines with duration of pupal diapause (Denlinger, 1981), thus it is advantageous for females to minimize diapause duration. Males and females emerge from diapause at the same time; the difference in duration simply reflects later diapause entry by the females. Sex differences are not noted in *Poecilometopa spilogaster,* a species from Kenya (Denlinger, 1979), suggesting there is no cost differential between males and females for entering diapause in this tropical location.

9. A maternal effect that blocks diapause

A conspicuous diapause maternal effect is noted in *S. bullata*, and perhaps in some, but not all, other temperate latitude sarcophagids (Henrich & Denlinger, 1982a). Females that have experienced an overwintering diapause produce offspring that are incapable of entering diapause even when subjected to strong diapause-inducing signals of short daylength and low temperature. The male's diapause history is not important. From an ecological perspective, the maternal effect makes great sense. This block enables females to emerge in early spring without jeopardizing premature diapause entry by their progeny in response to the short daylengths prevailing at that time. By the time that first generation is completed, the days are long and would not lead to diapause. After completing a nondiapause generation, the flies can again respond to short days for programming diapause entry in the autumn. The response is dependent not upon the diapause experience itself but by the mother's short-day exposure prior to entry into diapause although these two features normally coincide. Although diapause is averted by the maternal effect, the progeny retain some features intermediate between the diapause and nondiapause phenotypes: length of larval wandering, fecundity, and quantities of puparial hydrocarbons are intermediate between the two phenotypes (Rockey et al., 1991).

The maternal information is retained in the larval brain and transferred to the ovaries sometime before the third day of the female's adult life (Rockey et al., 1989). Nerve transections and administration of juvenile hormone or ecdysteroids do not alter the maternal effect. Several agents can, however, influence the maternal effect. Rearing larvae on a diet supplemented with deuterium oxide (heavy water) subverts the blockage of diapause (Webb & Denlinger, 1997), presumably by interfering with the normal timekeeping mechanism. In addition, both gamma-aminobutyric acid (GABA) and one of its antagonists, picrotoxin exert opposite effects on the maternal effect: GABA suppresses the incidence of diapause in the female's progeny, while picrotoxin increases the diapause incidence (Webb & Denlinger, 1998). These results suggest that a GABA-mediated response within the mother is involved in regulating the maternal effect. Distinct microRNA profiles are also associated with the maternal effect in *S. bullata* (Reynolds et al., 2017), suggesting involvement of yet another layer of control.

10. What environmental features bring diapause to an end?

Photoperiod plays no role in diapause termination in the sarcophagids, nor is a period of chilling required for termination (Denlinger, 1972a). Chilling, however, can accelerate the process. A fixed period of time must elapse before diapause can be broken. Diapause will break spontaneously, and it does so more rapidly at higher temperatures. In the field, a diapause initiated in late summer or autumn must persist at least until the arrival of low winter temperatures. The normal progression for *S. bullata* from 40°N (Illinois) is for diapause to be initiated during late summer and autumn, pupae complete their true diapause by early January and then are fully competent to develop into adults but fail to do so until higher temperatures return in the spring (Denlinger, 1972b). This period after they are competent to develop but fail to do so because temperatures are too low is referred to as postdiapause quiescence. In the *S. bullata* example from Illinois, pupae entering diapause in August and September are fully competent to reinitiate development (enter postdiapause quiescence) by early January, but development in the field is delayed until early April when postdiapause quiescence is ended and pharate adult development begins, ending in emergence of adults in mid-May. A similar role for chilling is noted as well for diverse temperate populations of *S. similis* and *S. peregrina* from Japan (Moribayashi et al., 2021).

Temperature also appears to be the dominant feature contributing to diapause termination in flesh flies from tropical Africa. As in temperate latitude species, spontaneous termination of diapause occurs later at low temperatures. For example, diapause in a Kenyan population of *Poecilometopa spilogaster* lasts 72 days at 18°C, 101 days at 15°C, 234 days at 12°C (Denlinger, 1974). But, if diapausing pupae held at 15°C for 10 days are transferred to 25°C, initiation of pharate adult development ensues within 4 days, demonstrating that the pupal diapause in these tropical flies is much more labile than in pupae from temperate regions. Experiments with *S. ruficornis*, a species from Brazil, show that cool daytime temperatures produce not only a higher incidence of diapause than cool night temperatures, but also a diapause of longer duration (Denlinger, 1979).

11. Molecular signaling pathways

All evidence points to a shutdown in the brain-prothoracic gland axis as a central controlling feature of pupal diapause in the sarcophagids, as it is in most other diapausing pupae (Denlinger et al., 2012). The ecdysteroid titer is low in diapause-destined pupae (Walker & Denlinger, 1980; Richard & Saunders, 1987; Moribayashi et al., 1988), ecdysteroid levels rise at diapause termination, and an injection of ecdysteroids readily terminates diapause (Zdarek & Denlinger, 1975; Denlinger, 1979). The diapause program in *S. crassipalpis* can be transferred from one individual to another by transplantation of the brain and ring gland (site of ecdysteroid synthesis), and an intact brain and ring gland is needed for successful diapause termination (Giebultowicz & Denlinger, 1986). Thus all results consistently underscore the critical role of the brain and prothoracic gland in diapause regulation in flesh flies.

Prothoracicotropic hormone (PTTH), the brain-derived neuropeptide that stimulates the prothoracic gland component of the ring gland to produce ecdysone, is present in brains of both diapause and nondiapausing pupae of *S. argyrostoma* (Richard & Saunders, 1987), and PTTH levels may even be higher in diapausing pupae as seen in *S. peregrina* (Moribayashi et al., 1992). This result suggests that PTTH is synthesized by both types of pupae but is simply not released in pupae destined for diapause. But, the shutdown in the brain-prothoracic gland axis is more than a failure of brain neurosecretory cells to release PTTH. The prothoracic glands of *S. argyrostoma* are also refractory to PTTH stimulation in diapausing pupae (Richard & Saunders, 1987), providing double assurance that development will be halted. The ring glands lose competency to synthesize ecdysone 1–2 days after the onset of diapause but quickly regain competency at diapause termination.

Juvenile hormone (JH) also appears to contribute to the diapause response in flesh flies, but it does not directly affect the diapause decision. It does, however, play a role in regulating the cycles of oxygen consumption (discussed above) that persist throughout diapause (Denlinger et al., 1984; Denlinger & Tanaka, 1989). JH may also contribute to diapause termination. Although JH by itself does not terminate diapause, a small spike of JH activity is noted prior to the rise in ecdysteroid levels at diapause termination (Walker & Denlinger, 1980), and a combination of JH and ecdysteroids is much more effective in terminating diapause than ecdysteroids alone (Denlinger, 1979).

Insulin signaling is emerging as a common theme involved in many insect diapauses, regardless of the stage (Denlinger, 2022). Although this signaling pathway has not been examined extensively in flesh flies, a transcriptomic comparison between diapausing and nondiapausing pupae of *S. crassipalpis* reveals numerous distinctions in expression levels for components of the insulin signaling pathway (Ragland et al., 2010). Other players are also likely involved in regulating pupal diapause in flesh flies. For example, the transcript encoding neuropeptide-like precursor 4 (Nplp4), a peptide precursor of unknown function, is highly upregulated in association with diapause in *S. crassipalpis* (Li et al., 2009a). Inos, myo-inositol-1-phosphate synthase, is also of potential interest for the diapause of *S. crassipalpis* (Li et al., 2009b). Inos plays a role in numerous signal transduction pathways in mammals, and its rapid increase at diapause termination in flesh flies suggests it may contribute to early events linked to the reinitiation of development.

Epigenetic mechanisms, those that influence gene expression without altering DNA sequences, are widely used by organisms to sense and respond to changing environmental conditions, thus it is not surprising to see evidence that such mechanisms may be linked to generation of the diapause phenotype (Reynolds, 2017). One such mechanism, histone modification, appears to be important for the diapause response in *S. bullata* (Reynolds et al., 2016).

Histone modification is evident not only in comparisons of diapausing and nondiapausing pupae, but distinct patterns of acetylation and deacetylation are evident in the photosensitive first-instar larvae, as well as in flies exhibiting the maternal effect.

Like epigenetic processes, small noncoding RNAs (sncRNAs) are relatively new to the stage of diapause regulation, but their role in regulating all sorts of developmental processes, including diapause, will likely be pervasive. In *S. bullata,* several components involved in sncRNA biogenesis have distinct diapause and nondiapause profiles, during the stage of diapause, as well as during the photosensitive stage and in relation to the diapause maternal effect (Reynolds et al., 2013). miRNAs, one category of sncRNAs, have huge numbers of targets, making it a challenge to precisely link expression with specific functions, but what is clear is that numerous miRNAs exhibit distinct expression patterns associated with diapause, including the diapause of *S. bullata* (Reynolds et al., 2017). Two additional classes of sncRNAs, the small interfering RNAs (siRNAs) and piwi-associated RNAs (piRNAs) have also been implicated as likely players in the diapause of *S. bullata* (Reynolds et al., 2013). Clearly this nascent field will grow in importance as we more fully probe the regulatory mechanisms undergirding diapause.

12. Inheritance of the diapause response

Selection experiments with *S. bullata* (Henrich & Denlinger, 1983), *S. similis* (Goto, 2009), and *Poecilometopa spilogaster* (Denlinger, 1979) demonstrate that the diapause response can be lost within a few generations. Multiple attributes of the diapause response appear to be linked, as revealed in *S. bullata.* Selection for a diapause-associated trait, such as a longer wandering period in the third instar, results in a higher diapause incidence and a diapause of longer duration (Henrich & Denlinger, 1982b). A similar linkage between diapause and larval wandering time is noted in *S. similis* (Goto, 2009): a line selected for low diapause also wanders for a much shorter time.

A simple Mendelian inheritance pattern is noted when diapausing and nondiapausing strains of *S. bullata* are crossed (Han & Denlinger, 2009b), and the pattern appears to be autosomal (Henrich & Denlinger, 1983). Polygenic inheritance patterns are much more commonly noted for diapause (Denlinger, 2022), and it is quite likely that a more sophisticated analysis involving quantitative trait loci will indeed reveal that the inheritance pattern for diapause in flesh flies is more complex than noted with simple crossing experiments. Yet, the results to date suggest that relatively few loci are likely to be involved.

13. Subverting diapause

Although pupal diapause in flesh flies is programmed rather early during embryonic and larval life, the program can be derailed, thus preventing "diapause-programmed larvae" from actually entering into diapause as pupae. For example, subjecting wandering third instar larvae to high temperatures or physically shaking the larvae can prevent diapause entry (Denlinger et al., 1988), and certain chemical agents such as cholera toxin (a cyclic AMP generator) can also prevent diapause entry (Denlinger, 1976). The efficacy of these diapause-preventing manipulations demonstrate that the programming of pupal diapause can indeed be subverted with certain stresses, suggesting that the fly can opt out of entering diapause at the last minute if subjected to certain adversities. By rearing larvae on a diet laced with heavy water (deuterium oxide) diapause can also be prevented (Rockey & Denlinger, 1983), an effect likely elicited by disruption of the timekeeping mechanisms critical for monitoring daylength.

Once diapause has been entered, a number of chemical agents can bring flesh fly diapause to an end and initiate adult development. As one would expect, ecdysteroids are particularly good hormonal agents for terminating diapause (Ždárek & Denlinger, 1975), and interestingly, combining juvenile hormone (JH) with ecdysteroids is even more effective. Although the secondary messenger cyclic AMP is effective in blocking diapause entry, it has no effect in terminating diapause, while cyclic GMP and its analogs are effective in breaking diapause, especially in combination with ecdysteroids (Denlinger & Wingard, 1978). This result suggests some interesting distinctions in the regulatory schemes for initiating and terminating diapause. Although JH by itself will not break diapause it can significantly shorten the duration of diapause (Denlinger et al., 1988). This effect is likely due to the elevated metabolic rate elicited by JH (Denlinger et al., 1984), causing the diapausing pupae to prematurely deplete its energy reserves. This implies an energy sensing mechanism that the fly uses to monitor and parse its energy reserves so that it not only can bring diapause to an end at the appropriate time but to also have sufficient reserves in store to complete pharate adult development, culminating in adult emergence.

By using acetone as a carrier for JH, we discovered that acetone itself is capable of breaking pupal diapause (Ždárek & Denlinger, 1975). This led to the testing of a wide range of chemical solvents, many of which are much more effective than acetone (Denlinger et al., 1980). Among the agents tested, hexane and di-ethyl ether are among the most effective, and both agents not only break diapause but also allow adults to emerge without apparent detrimental effects. These solvents can exert their effect either by topical application or vapor exposure. This hexane effect offers a useful tool for simultaneously breaking diapause in large numbers of pupae, thus making it possible to generate postdiapause cohorts of precise and uniform ages.

Physical manipulations known to break pupal diapause in *S. crassipalpis* include anoxia (Kukal et al., 1991) and heat shock (Denlinger et al., 1980). Faced with challenges of this sort that would ultimately prove lethal, the diapausing pupa can apparently make the decision to reinitiate development and thus escape certain death.

The potential disruption of the diapause response by artificial light at night (ALAN) and urban warming is a concern for all insects, and this issue has been nicely demonstrated in *S. similis* (Mukai et al., 2021). Caged flies reared outside in urban areas in Osaka, Japan, enter diapause nearly four

weeks later than caged flies in nearby rural areas. In the warm urban area with abundant artificial light many flies fail to enter diapause, even in late autumn. The delay is more pronounced in females than in males.

14. Why sarcophagids are not always ideal for the study of diapause

In spite of the extensive database available for the sarcophagids, they are not in all ways ideal for diapause studies. The major current drawback is that RNA interference (RNAi) has only been modestly effective on these flies. Although knockdown of genes encoding the heat shock proteins has been possible (e.g. Rinehart et al., 2007), many other gene targets have not been successfully suppressed using RNAi. This issue may, of course, be moot if the more powerful knockdown technique of CRISPR/Cas 9 proves effective in flesh flies.

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