

Trends in Genetics



Complications of complexity – diapause in insects

Understanding sex chromosome dosage compensation

Orthologs and paralogs

Thirty years of *Mus spretus* research

Complications of complexity: integrating environmental, genetic and hormonal control of insect diapause

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Understanding gene interaction and pleiotropy are long-standing goals of developmental and evolutionary biology. We examine the genetic control of diapause in insects and show how the failure to recognize the difference between modular and gene pleiotropy has confounded our understanding of the genetic basis of this important phenotype. This has led to complications in understanding the role of the circadian clock in the control of diapause in *Drosophila* and other insects. We emphasize three successive modules – each containing functionally related genes – that lead to diapause: photoperiodism, hormonal events and diapause itself. Understanding the genetic basis for environmental control of diapause has wider implications for evolutionary response to rapid climate change and for the opportunity to observe evolutionary change in contemporary time.

Genes and modules

Mapping genotype to phenotype, understanding how genes interact with other genes, and how genes can affect multiple phenotypes (pleiotropy) have been long-standing goals of developmental and evolutionary biology. It has been recognized for many years that organisms tend to be modular, insofar that they are composed of developmental or physiological processes that are tightly integrated into units operating to a certain degree independently of other processes. This basic concept of modularity dates back at least to Herbert Simon [1], who argued that genes can be integrated into functionally autonomous groups. Modular pleiotropy (see Glossary) can then be defined as the effect of a module on multiple phenotypes, being distinct from gene pleiotropy, which defines the typical effect of individual genes on multiple phenotypes. Therein lies the complication of complexity because the fact that a gene constitutes a component part of a functional module does not preclude that gene from having a separate and important ancillary function outside of the module. Modularity has been extensively considered in developmental pathways, especially those involved in cell signaling such as Hedgehog [2], transforming growth factor (TGF) β [3], Wnt [4] and Notch [5], in which investigators usually recognize the distinction between modular and gene pleiotropy; however, this distinction is less well appreciated in physiological genetics. Herein, we examine genetic control of a physio-

logical trait, using insect dormancy (diapause) as an illustrative example, and show how not recognizing the difference between modular and gene pleiotropy, especially when bound by long-standing historical expectations, has confounded rather than clarified the genetic basis of this important phenotype.

Physiological processes represent the connection between the genome and the external world. Approximately two-thirds of the world's land mass lies within temperate and polar regions of the earth. Within these regions, the majority of insects escape the exigencies of winter through

Glossary

Circadian clock: the circadian clock is an endogenous, internally maintained biochemical oscillation with a period of approximately a day. Circadian rhythms consist of two elements, a period and an amplitude of oscillation, without which there is no rhythm. The circadian clock is involved in the daily control of a diverse array of biochemical and physiological events.

Clock genes: clock genes are involved in the core functioning of the circadian clock. *period* and *timeless* form a negative feedback loop of the *Drosophila* clock; *cycle* and *clock* form a positive feedback loop; and *cryptochrome* connects the clock with light (Figure 2).

Critical photoperiod: the critical photoperiod is the length of day or night eliciting an equal percentage of diapause and development; the inflection point of the photoperiodic response curve.

Diapause: diapause is a hormonally controlled cessation of development that is associated with stress tolerance and increased lipid sequestration. Diapause enables arthropods to mitigate the negative effects of harsh seasons.

Forward genetic approach: forward genetics seeks to find the genetic basis of a phenotype or trait; that is, to identify mutations or naturally segregating alleles that produce a certain phenotype. Examples of this approach include fine-scale QTL mapping, expression microarrays, and bulked segregant analysis. This approach contrasts with reverse genetics, which determines the phenotype that results from mutating a given gene.

Gene pleiotropy: gene pleiotropy occurs when a single gene affects more than one phenotype, the classic example being sickle cell anemia, in which a single nucleotide substitution results in reduced oxygen transport by hemoglobin but also increased resistance to malaria.

Interval timer: an interval timer assesses the length of day or night through processes not causally dependent on the circadian clock (e.g. the vetch aphid, *Megoura viciae* [76,77] or the photoperiodic timer in the spider mite *Tetranychus urticae* [78]).

Modular pleiotropy: modular pleiotropy describes when a gene or genes have multiple phenotypic effects only indirectly by modifying the functional expression of the module in which they have a role.

Module: a module is group of functionally related genes that interact with each other to control integrated processes, such as photoperiodism. Usually, genes within a module interact with each other more than they do with genes in other modules.

Photoperiodic response curves: a photoperiodic response curve is a plot of diapause or its converse, development, as a function of day length. It is usually sigmoid in form. From these curves, one can calculate the critical photoperiod of the transition between diapause and development.

Photoperiodic response: the photoperiodic response is a phenotypic response to day or night length; herein, the photoperiodic response is diapause or development.

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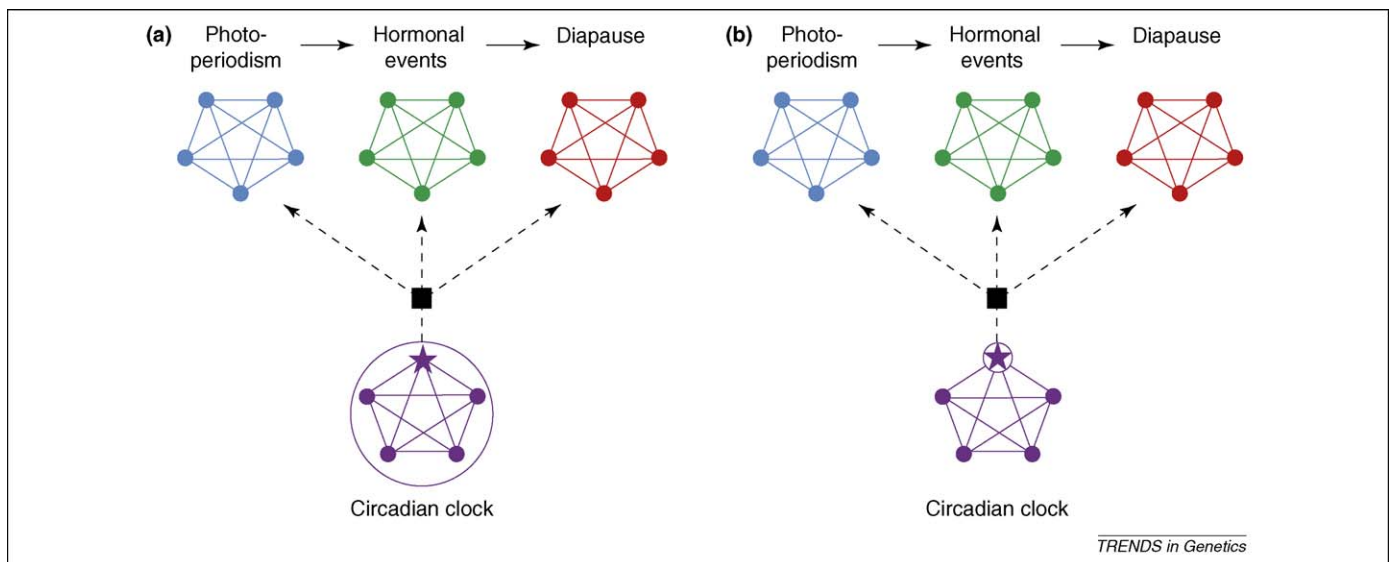


Figure 1. Schematic illustration of genetic events leading to diapause. Each pentagram represents a module of functionally related genes. The photoperiodism (blue)–hormonal event (green)–diapause (red) axis shows sequential events that are established and accepted. The role of the circadian clock (purple) in affecting diapause remains uncertain and is the primary subject of this figure. **(a)** Modular pleiotropy. A mutation of a clock gene (★) modifies the function of the circadian clock and this modified clock function then affects the expression of diapause. The circadian clock might potentially exert its control through photoperiodism or hormonal control of diapause, or might act on diapause directly, as indicated by the broken lines through unknown connections (black square). **(b)** Gene pleiotropy. A mutation of a clock gene (★) might or might not affect clock function but does affect the expression of diapause, independently of its role in the circadian clock. This single gene might exert its control through photoperiodism or hormonal control of diapause, or might act on diapause directly, as indicated by the broken lines through unknown connections (black square). Distinctions between gene and modular pleiotropy and knowledge of where in pathways mutations have their effects is crucial for understanding the genetic basis of phenotypes.

dormancy or migration, which is initiated by a physiological response to day length called photoperiodism [6–10]. Seasonality, in turn, is a reflection of latitude and altitude [11]. In response to recent rapid climate change, both plants and animals have expanded their ranges northwards and have altered the pattern of their seasonal activities [12–17]. Perhaps not surprisingly, the first evidence for an evolutionary response (genetic change) to recent climate warming over a geographic gradient involved a genetic shift towards shorter, more southern day lengths controlling diapause [18,19], thereby making this phenotype one of the most powerful diagnostic measures of the global threat of a warming world. Hence, examining the genetic basis for environmental control of diapause is particularly timely because of the societal implications of biotic response to rapid climate change and because of the opportunity to observe evolutionary change in contemporary time.

Understanding the genetic foundations of insect diapause and other complex adaptations will largely be gained by using forward genetic approaches (proceeding from phenotype to genotype), by matching the appropriate organism with the appropriate phenotype, and by probing the genetic basis of natural phenotypic variation over geographical gradients.

The structure of the photoperiodism–hormone–diapause axis

Many insects have evolved a strategy called diapause – a stage of developmental arrest – that enables them to survive harsh seasonal conditions, such as extremes in temperature or drought. Diapause can occur at any stage of the life cycle, from embryonic to adult reproductive dormancy. A wide variety of arthropods living at temperate

and polar latitudes use the length of day (photoperiodism) to initiate a cascade of hormonal events that culminate in diapause (Figure 1). This photoperiodic response incorporates the input of light, a timer that assesses the length of day or night, a counter that accumulates the number of long- or short-day signals received, and an output signal to the hormonal control of diapause [6]. Each of these processes from the input of light to the output signal, and the genes controlling them, interact to elicit photoperiodic response. Therefore, photoperiodism can be considered as a module, which we define as a group of functionally related genes that interact with each other to control integrated processes. Usually, genes within a module interact with each other more than they do with genes in other modules. Nonetheless, individual genes within a module can have important ancillary effects that are independent of their role within a given module.

When the photoperiodism module produces an output signal that ultimately results in diapause, the hormones involved in diapause must be synthesized, secreted and degraded at the appropriate rates and times. Hence, the hormonal control of diapause can be considered as a second module between photoperiodism and diapause itself (Figure 1). Finally, output from the endocrine system elicits a syndrome of diapause traits that includes the cessation of active development, reproductive dormancy, the accumulation and sequestration of energy reserves, increased stress resistance, reduced metabolic rate or a combination of these traits. Diapause is then the final module in the photoperiodism–hormone–diapause axis.

The distinction between an individual gene and a module is important in complex traits because, regardless of its exact definition, gene actions are often perceived as part of functionally integrated processes (modules).

Understanding the modular structure of processes has greatly advanced our knowledge of how a genotype becomes a phenotype [20]. However, undue focus on modularity leads to the logical temptation to conclude that the effects of a single mutant gene in the laboratory or a single allele segregating in natural populations must have its effect through its principal module and that the alteration of the entire module determines the causal change in the phenotype of interest. This conclusion is tantamount to assuming that a gene cannot have an effect outside of its principal module; however, effects of single genes on multiple phenotypes (pleiotropy) are very common [21]. Likewise, modules themselves can have pleiotropic effects [22]. It is the failure to distinguish between gene and modular pleiotropy that complicates interpretation of genetic mutations in laboratory lines or interpretation of allelic variation segregating in natural populations.

The role of the circadian clock in diapause

The circadian clock controls daily timing at the molecular, cellular, physiological and behavioral levels in organisms from Cyanobacteria to mammals [23]. The circadian clock might be considered as a fourth module potentially affecting diapause (Figure 1). Since Bünning's influential hypothesis [24] that circadian rhythms formed the basis (*Grundlage*) of photoperiodism, investigators have sought physiological parallelisms between circadian behavior and photoperiodic response. With a deeper understanding of the molecular basis of circadian rhythmicity in *Drosophila melanogaster*, circadian rhythm genes quickly became candidate loci for photoperiodism. However, a distinction needs to be made between Bünning's *Grundlage* that the circadian clock forms a causal, necessary basis of photoperiodism and the concept that the circadian clock can influence the photoperiodic response. Given the pervasive effects of the circadian clock on cellular biochemistry [25], it would be surprising to find any physiological function that is not affected in some way by the circadian clock. Mutation or allelic variation in genes involved in circadian rhythmicity could affect diapause by modifying clock function so that the circadian clock as a module would alter photoperiodism, hormonal control or diapause itself (Figure 1a). Alternatively, mutation or allelic variation in a clock gene could affect diapause directly as an individual gene, independently of its role in the circadian clock (Figure 1b). There are two known clock genes with a role in photoperiodic Diptera that illustrate this distinction.

The genes *period* (*per*) and *timeless* (*tim*) code for the proteins PERIOD and TIMELESS that form an integral part of the negative regulatory loop of the circadian clock of *D. melanogaster* (Figure 2). If the circadian clock, as a module, has a causal role in photoperiodism, laboratory mutants or naturally segregating alleles of these loci should alter both circadian function and photoperiodic response.

Clock genes and diapause

The wild-type Canton-S strain of *D. melanogaster* (isolated c.1930 near Canton, Ohio, USA [26]) is photoperiodic for delayed ovarian maturation (diapause) over a restricted range of moderately low temperatures in the laboratory

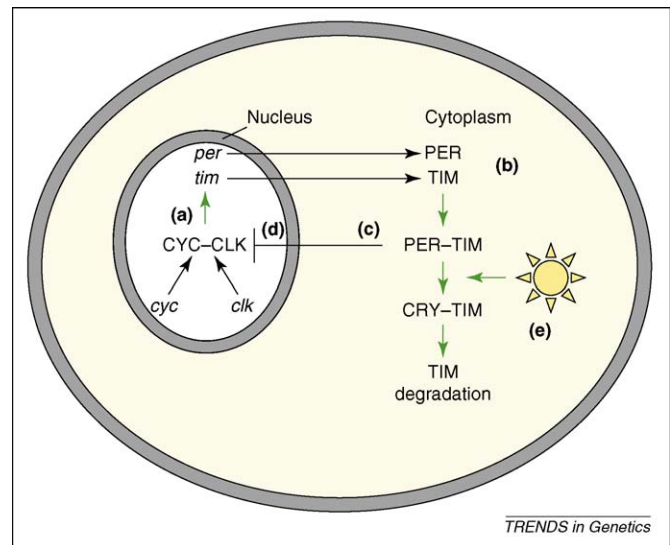


Figure 2. Simplified schematic of the *period-timeless* feedback loop in the *Drosophila* circadian clock. (a) The CLOCK-CYCLE (CYC-CLK) heterodimer is a transcription factor that promotes the transcription of *period* and *timeless* (*per* and *tim*) mRNA. (b) *per* and *tim* mRNAs are transported to the cytoplasm in the early evening where they are translated into their respective proteins, PERIOD (PER) and TIMELESS (TIM). After a delay of a few hours, PER and TIM are translocated back into the nucleus (probably as a dimer) in the late evening. (c) Once in the nucleus, PER-TIM might dissociate, and PER physically disrupts the transcription factor CLK-CYC, leading to a fall in *per* and *tim* levels. (d) Nuclear TIM and, later, PER are then degraded, and this degradation enables the derepression of *per* and *tim* promoters via CLK-CYC, so the molecular cycle starts again with *per* and *tim* transcription late in the day. (e) In the presence of light, the protein CRYPTOCHROME (CRY) binds TIM, preventing its dimerization with PER and leading to its phosphorylation and degradation. This cycle continues in constant darkness but is amplified in light-dark cycles, when the blue light receptor CRY is activated. CRY physically interacts with TIM and the F-box protein JETLAG, and TIM is phosphorylated and degraded [34]. When the PER-TIM heterodimer is accumulating in the early evening, light retards the accumulation of the PER-TIM dimer and sets the clock back. When PER-TIM is degrading late in the night, light accelerates this breakdown and sets the clock ahead.

[27,28]. Initially, three mutants were discovered that altered clock function: *per*⁰¹, *per*^s and *per*^l (later, *per*^{L1}), which eliminate, shorten or lengthen the period of circadian rhythmic activity or eclosion, respectively [29,30]. Saunders and colleagues [28,31] determined the photoperiodic response curves for these mutants and an additional mutant, *per*⁻, a double deletion of the *per* locus (Figure 3). All four mutants, including the arrhythmic *per*⁰¹ and *per*⁻ mutants, exhibited robust photoperiodic response curves, leading them to conclude that the *period* locus was not causally involved in photoperiodic time measurement and that the crucial genes were to be found elsewhere in the genome [28]. These results mean that neither *per* nor a functional circadian clock module is necessary for photoperiodic response.

The results from Saunders' experiments with *per* mutants led multiple laboratories, including our own [32,33], to investigate genes that function in the pathway between the input of light and the feedback loop involving PER (Figure 2). Both circadian rhythmicity and photoperiodism are triggered by light and it is the interaction of light, TIM and CRYPTOCHROME (CRY) proteins that sets the circadian clock. In the presence of light, CRY binds TIM, whereupon TIM is phosphorylated and degraded [34]. In *per* null mutants, *tim* is transcribed continuously and is translated into the TIM protein. In the presence of light, TIM still binds to CRY and is ultimately degraded; in the

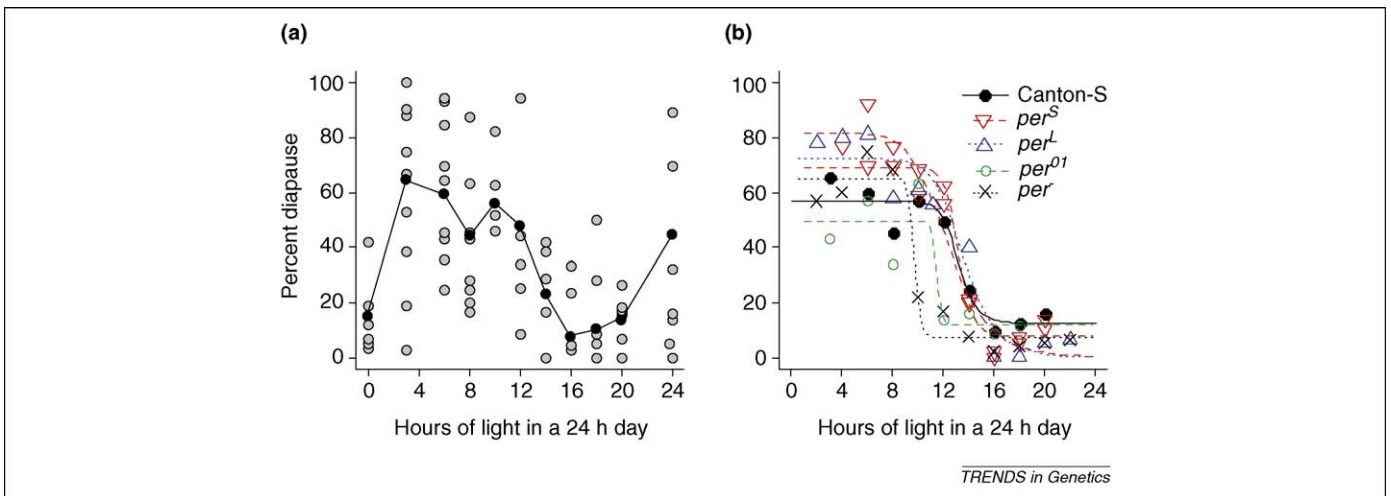


Figure 3. Photoperiodic response of *D. melanogaster* and *period* mutants. (a) Photoperiodic response of wild-type Canton-S (redrawn, with permission, from Ref. [28]). Light gray circles represent replicate groups; solid black circles represent group means. (b) Photoperiodic response of Canton-S wild type compared with various *per* mutants. Explanation of terms: *per^S*, two replicates of a short period line; *per^L*, a long period line; *per⁰¹*, an arrhythmic line; *per⁻*, double overlapping deletion line lacking the *per* gene altogether. Data points represent means of groups at that day length [31] (adapted, with permission, from Sage Publications). We fit the lines by logistic regression [79], omitting day lengths of 0 and 24 h because all lines were not represented at these day lengths. Critical photoperiods and their standard errors were determined from logistic regression. After applying sequential Bonferroni [80] to account for *a posteriori* multiple comparisons, we found no significant differences in critical photoperiods among lines, either using all possible comparisons ($P \geq 0.40$) or comparing each mutant line against only wild-type Canton-S ($P \geq 0.43$). These comparisons mean that there are no significant differences in left-right position of the sigmoid curves. Means and standard errors of the critical photoperiods are provided in the supplementary material online.

absence of light, TIM continues to accumulate. Hence, even in the absence of a functional circadian clock, the TIM protein continues to cycle in proportion to day length [35,36]. TIM could then function as an interval timer and provide the downstream cue for hormonal control of diapause. In the only quantitative trait loci (QTL) map for photoperiodism in animals (the mosquito, *Wyeomyia smithii*), *tim* does not fall under any of the 6–9 QTL identified so far for photoperiodic response, although *tim* or a closely linked gene interacts epistatically with six other markers on the third chromosome that affect the evolution of photoperiodism [33]. The evolution of photoperiodic response in North American *W. smithii* is not correlated with either the period or amplitude of the circadian clock [37–39]. These results are consistent with *tim* acting through gene, and not modular pleiotropy, to influence, but not to provide the basis for, the evolution of photoperiodic time measurement in *W. smithii*.

timeless is also involved in diapause of the Japanese drosophilid *Chymomyza costata*. *C. costata* enters a larval diapause that is mediated by photoperiod at 18 °C [40]. A non-diapausing mutant, *npd* (for non-photoperiodic diapause), was isolated from a recently collected population south of Sapporo. The *npd* mutant is arrhythmic for adult eclosion and the *npd* larvae lack daily and circadian oscillations of *per* and *tim* transcription [41,42]. Ultimately, *npd* was traced to the *tim* locus [43]. Compared with wild type, the *npd* mutant has an 1855 base-pair deletion in the 5'UTR and promoter regions, which removed the start of transcription, and all the regulatory motifs found in the wild-type flies. The *npd* mutant also included 37 non-synonymous substitutions in five of the 11 exons, including the PER interaction domains, five amino acid substitutions in the cytoplasmic localization domain, and one in the stop codon. Clearly, *tim* function in *C. costata* is necessary for the expression of circadian behavior and for diapause, but

it is not clear whether the effect of *tim* on diapause is exerted through the circadian clock (modular pleiotropy) or independently (gene pleiotropy) through interaction with photoperiodism, with the hormonal control of diapauses, or with diapause itself.

European populations of *D. melanogaster* are photoperiodic for the regulation of ovarian diapause [44]. Within European populations, *tim* is polymorphic for transcribing an ancestral short form of *tim*, *s-tim*, or both a long and short form, *ls-tim* [45]. In both Italian and Dutch populations, there is a linear decline in the incidence of diapause with increasing day length and, at each day length, the incidence of diapause is greater in *ls-tim* than in *s-tim* flies [44]. However, the photoperiodic response curves of both Italian and Dutch populations are linear and parallel. Hence, there is no significant *tim* genotype by day-length interaction. In transgenic flies with a common genetic background, the derived L-TIM protein binds less tightly to CRY than the ancestral S-TIM protein, thereby inhibiting the breakdown of TIM by light and rendering the circadian clock less sensitive to light [46]. The *l-tim* transgenics exhibit a higher incidence of diapause than the *s-tim* transgenics and, again, there is no genotype by day-length (short versus long days) interaction. In the natural populations and the transgenics, the lack of a genotype by day-length interaction means that *tim* and day length have additive and independent effects on the incidence of diapause (Figure 4). The effect of *tim* genotype on sensitivity of the circadian clock [46] then means that *tim* presents a clear case of gene but not modular pleiotropy, either through hormonal control or by affecting diapause directly.

A consideration of *period* and *timeless* illustrates the risk in assuming that because a gene has a major role in a given module, the effect of that gene must be due to modular and not gene pleiotropy. Neither mutants of *per* nor natural allelic variation in *tim* provide a clear demon-

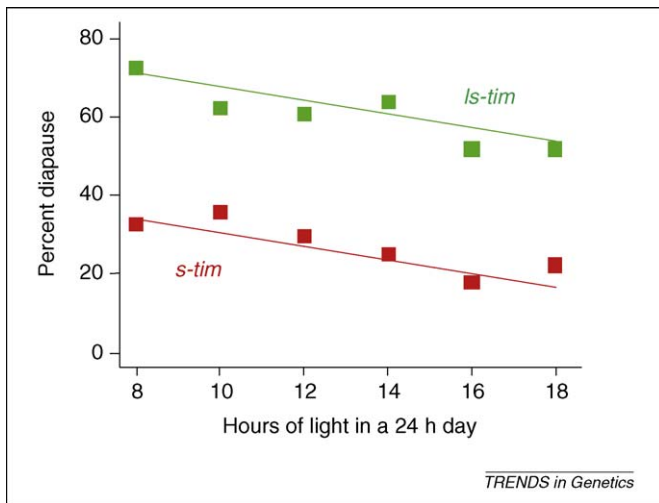


Figure 4. Photoperiodic response of *D. melanogaster* from Salice, Italy. Analysis of covariance (ANCOVA) shows no significant day length by *timeless* genotype interaction: the regression lines for *ls-tim* and *s-tim* flies are parallel. The parallel lines indicate that an increase or decrease in day length by 2 h has the same effect on diapause in both the *ls-tim* and *s-tim* lines; or, the effect of a *timeless* allele on diapause is the same regardless of photoperiod. Hence, the two factors, day length and *timeless* allele, are acting additively and independently. Figure adapted, with permission, from Ref. [44].

stration that the circadian clock as a functional module is responsible for or contributes to diapause expression in adult flies.

Diapause and hormonal control of ovarian maturation

Operationally, adult diapause in *D. melanogaster* is scored by ovarian development, which ceases or at least slows substantially at the pre-vitellogenic state (before yolk-protein uptake by the oocyte) in diapausing individuals over a narrow range of intermediate temperatures [28]. Warmer temperatures invariably avert diapause; colder temperatures halt development at any stage. Furthermore, if maintained long enough at the diapause-inducing temperature (12 °C), ovarian maturation resumes after a delay of several weeks, even in the long-established photoperiodic Canton-S line [28]. Clearly, if one wants to study the effect of latitude or genetic variation on diapause, one selects the constant temperature at which a chosen effect on the incidence of diapause is greatest. For good measure, assays of diapause are run on short days, although day length has not been shown to affect the incidence of diapause in any natural population of *D. melanogaster* from North America. Not surprisingly, separate studies of diapause in populations of *D. melanogaster* have each been carried out at a constant moderately low temperature between 11 and 13 °C on a short day (10L:14D) with controls at a warm temperature on a long day.

Diapause in *D. melanogaster* is characterized by delayed vitellogenesis but also involves increased stress resistance and lipid sequestration [47]. The incidence of diapause increases with latitude in eastern North America [48,49] and in eastern Australia [50]. Diapause in *D. melanogaster* therefore represents a syndrome of traits expressed in a formerly tropical insect that seems to improve fitness in progressively more temperate seasonal environments.

A composite picture of hormonal control of ovarian maturation and diapause in Diptera is emerging from

studies on ovarian diapause in *D. melanogaster* and mosquitoes (Figure 5). Diapause is mediated by the insulin-signaling pathway (Figure 5). Insulin-like proteins (IIP) are secreted by the dorsal medial neurosecretory cell and travel down the recurrent nerve to the ring gland. This gland consists of three endocrine organs fused into one structure surrounding the esophagus: the prothoracic gland, which synthesizes and secretes ecdysteroids (steroid hormones); the corpora allata, which synthesize juvenile hormone (JH, a sesquiterpenoid lipid-like molecule); and the corpora cardiaca. The corpora cardiaca are neurohemal organs that release hormones into the hemolymph. In combination, the presence or absence of JH from the corpora allata and ecdysteroid from the ring gland and the ovaries control ovarian diapause [51–55].

In *D. melanogaster*, the insulin signaling pathway is involved in aging [56–59] and manipulations of the phosphatidylinositol 3 kinase (PI3K)–Forkhead box O (FOXO) cascade in *D. melanogaster* and mosquitoes result in phenotypes similar to diapause (Figure 5). Insulin-like peptides from the dorsal median neurosecretory cells in the brain promote yolk uptake and ecdysteroid production by the ovaries [60], lipid mobilization, increased fecundity, and shorter lifespan [57]. The insulin receptors are necessary for ovarian development; knockouts are rescued by JH in a dose-dependent manner [49,61,62]. The insulin receptor substrate (CHICO) is necessary for normal levels of JH synthesis [62] and PI3K is necessary for ovarian development [49,62]. FOXO controls lipid sequestration in diapausing adults and insulin-dependent signaling in the fat body, in addition to oxidative stress resistance and extended lifespan [61,63]. Finally, allelic variation in PI3K is associated with latitudinal increase in the incidence of diapause in North American *D. melanogaster* between Georgia and Ontario [49].

Together, these results and observations indicate that the insulin-like peptides, JH and ecdysteroids acting through the PI3K–protein kinase B (AKT)–FOXO cascade provide the pivotal hormonal connection between the environment and ovarian diapause in adult Diptera. The hormonal control of diapause thereby provides an integrated module that, through its pleiotropic effects, influences both the diverse traits associated with diapause and longevity, not only in mutant flies in the laboratory, but also in the geographically variable expression of diapause in natural populations.

Couch potato, linkage and co-adaptation

Couch potato (*cpo*) is so called because hypomorphic adults are lethargic, recover slowly from anesthesia, are reluctant to fly, and exhibit only weak positive phototaxis or negative geotaxis [64]. In eastern North America, the latitudinal cline in diapause is associated with increased stress and cold resistance and increased lipid storage [47,48]. Increasing diapause along the latitudinal cline is also associated with an increasing frequency of the derived substitution of lysine for ancestral isoleucine in residue 462 in the 3' end of exon 5 at this locus. *cpo* is expressed in the ring gland and has a series of upstream ecdysone response elements, suggesting that the effects of *cpo* on diapause in *D. melanogaster* is mediated by ecdysteroids [65]. Therefore, *cpo*

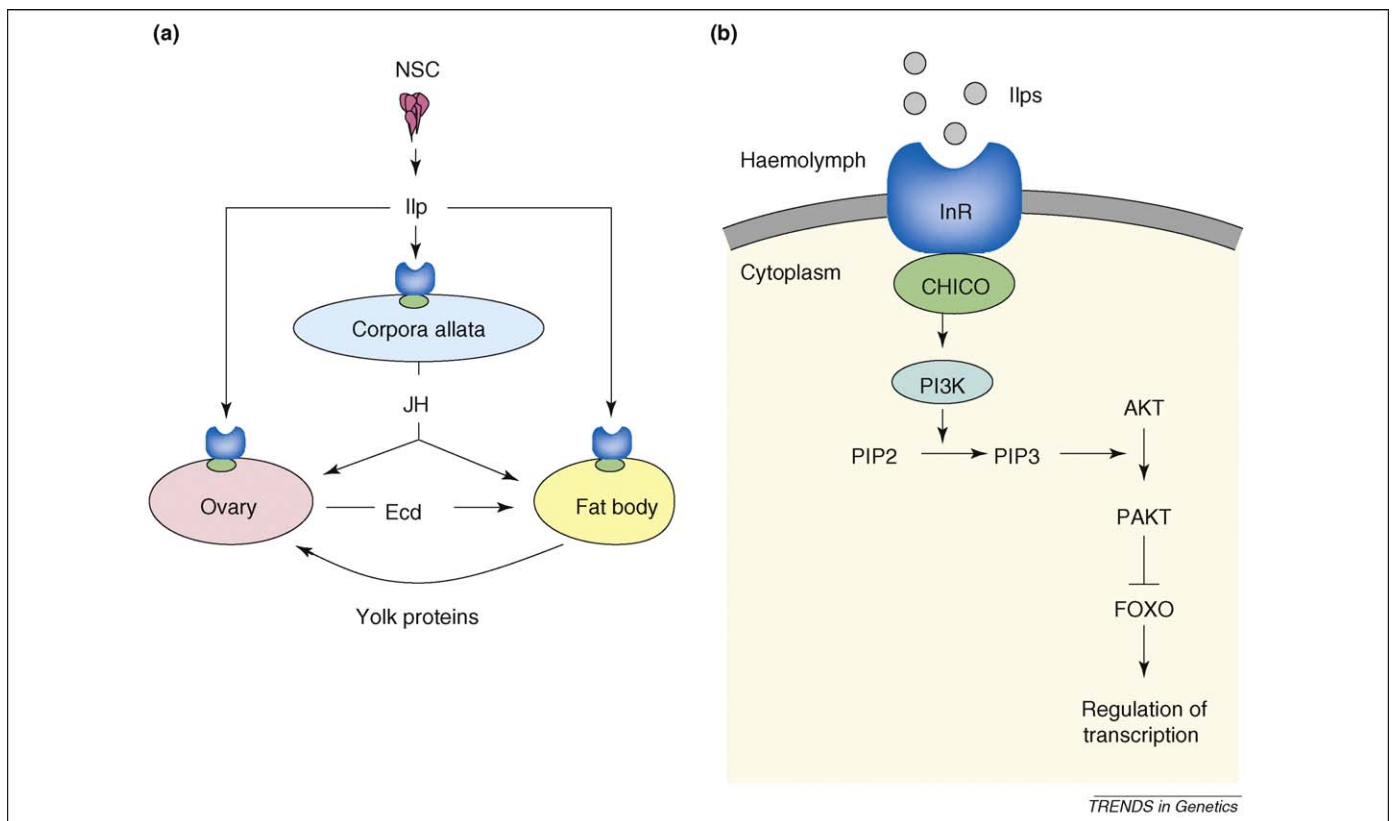


Figure 5. Endocrine control of ovarian maturation in flies (Diptera). **(a)** Major hormonal events. Upon appropriate environmental input, neurosecretory cells in the dorsal midbrain (NSC) secrete insulin-like peptides (Ilp) that stimulate the corpora allata (CA) in the ring gland to secrete juvenile hormone (JH). JH acts early in ovarian maturation to stimulate yolk protein synthesis in the ovaries and fat body. In response to JH, the ovaries secrete ecdysteroid (Ecd) that stimulates uptake of yolk protein. Ecd from the ovaries stimulates sustained yolk protein synthesis later in ovarian maturation. **(b)** The PI3K–FOXO cascade in the insulin signaling pathway. Ilps are bound by the insulin receptor (InR) and the insulin receptor substrate (CHICO) that, along with PI3K, phosphorylate phosphatidylinositol (4-5)-bisphosphate [PtdIns(4,5)P₂; PIP₂] to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P₃; PIP₃]. This, in turn, acts as a phosphate source to phosphorylate serine–threonine kinase (AKT→PAKT). PAKT then acts as a phosphate source to phosphorylate the forkhead transcription factor (FOXO), leading to its degradation. FOXO enters the nucleus where it regulates transcription. Compiled from Refs [52,54,58,59,81].

could provide the link between the insulin signaling pathway and the downstream hormones involved directly in the regulation of vitellogenesis.

Both *cpo* and *Pi3K* affect the incidence of diapause and are involved in latitudinal clines in propensity to diapause. These genes and the gene encoding the insulin-like receptor, *InR*, are located on the right arm of the third chromosome within the boundaries of a known inversion, *In(3R)Payne*. The frequency of *In(3R)Payne* declines with increasing latitude in both North America and Australia and shows strong linkage disequilibrium between marker loci within the inversion [66]. This linkage disequilibrium indicates that diapause-promoting alleles might be positively linked (coupled), forming a co-adapted gene complex that restricts recombination between diapause-prone winter alleles and diapause-averse summer alleles.

Concluding remarks

Our current understanding of the genetics of diapause illustrates both how historical inertia and the failure to distinguish between gene and modular pleiotropy has impeded progress in understanding the relationship between circadian rhythmicity, photoperiodism and diapause. The historical inertia created by Bünning's hypothesis that circadian rhythms constitute the basis of photoperiodic control of diapause led to a focus on circadian

clock genes as candidate loci for photoperiodism. Until recently [44], it has been assumed that mutations or allelic variants in clock genes that modify both overt rhythmicity and diapause have done so by modifying the effect of the entire circadian clock on photoperiodism, and not by having direct effects on diapause, independently of their role in modifying circadian rhythmicity (i.e. the failure to distinguish between modular and gene pleiotropy). Awareness of this distinction is important because, with the emergence of new molecular tools and an exploding genomics database, today's association, correlation, presumptive connection or logically plausible role, like Bünning's hypothesis, has a tendency to acquire a momentum of its own and become tomorrow's accepted assumption or established dogma.

Nonetheless, many years of careful research at physiological, evolutionary and genetic levels have resulted in a solid working framework for understanding reproductive diapause in insects. There is general agreement that input of light is interpreted by a timing mechanism that assesses the length of day or night, a counter that accumulates the number of long or short days received, and an output signal. The output signal, which is not yet known, initiates a cascade of hormonal events that ultimately results in diapause itself. The hormonal events are initiated by the release of insulin-like peptides from the brain and involve

sequential secretion of juvenile hormone by the corpora allata in the ring gland and ecdysteroid by the ovaries to promote yolk protein synthesis in the ovaries and fat body. Hence, the insulin signaling pathway is involved not only in aging but also in regulating diapause, both of which involve increased stress resistance, lipid sequestration and postponed senescence in adult Diptera.

On an evolutionary scale, diapause as a phenotype has provided unexpected opportunities to assess the impact of global warming on natural populations, on agriculture and on vector borne disease. Contemporary evolution is proceeding faster than was conceived a generation ago [67,68]. As the length of the growing season increases at temperate latitudes, there already have been genetic shifts towards more southern phenotypes in photoperiodic response and the timing of diapause, which have been observed over as short a time span as five years in mosquitoes and moths [18,69]. The frequencies of *In(3R)Payne* – the summer inversion – have increased over a latitudinal gradient during the past 20 years in Australian populations of *D. melanogaster* [70], suggesting that evolution of diapause in response to recent rapid climate change has involved not only photoperiodism but also downstream events associated with *couch potato* and at least one of the genes (*Pi3K*) in the insulin signaling pathway.

Future directions

Reverse genetic approaches require an *a priori* assumption of which genes are important to consider. The use of circadian clock genes as candidate loci has shed little light on the genetic basis of photoperiodism, whereas QTL mapping has identified 6–9 QTL contributing to the evolution of photoperiodism in a mosquito [33]. Even in a genetic system as well understood as the circadian clock in *Drosophila*, a new negative feedback loop involving *clockwork orange* was discovered through the use of an expression microarray [71,72]. Along with others [73–75], we therefore propose that forward genetic approaches such as fine-scale mapping, expression arrays, and bulked segregant analysis are fruitful avenues for gene discovery, even in systems that are considered to be well understood.

Reverse genetic approaches typically use induced mutations in cloned genes that are introduced into cells or the germ line and then observed for the phenotypic consequences. This procedure works well for discovering genes of major effect in model organisms but is less likely to identify genes with allelic variation undergoing selection in natural populations. A forward genetic approach starting with latitudinal variation in diapause ultimately led to the discovery of the role of *couch potato* in the regulation of diapause in *D. melanogaster* [48,65]. We therefore propose that more advantage be taken of natural geographic variation to reveal the genes responsible for adaptive evolution in the real world.

We are in agreement with others [28,53] that choice of the appropriate insect for the phenotype under consideration is essential. Although making a huge contribution to genetics, development and evolution, the weak photoperiodic response of *D. melanogaster* unfortunately makes it a lesser organism for the study of photoperiodism despite heroic efforts to do so [27,28,44]. There is a wealth of other

insects with clean, precise and robust photoperiodic responses [7,8,10] that would produce more definitive results. The increasing accessibility of molecular tools for non-model organisms makes the choice of the appropriate insect especially imperative.

Finally, we propose that when experiments are designed and data are interpreted, special care be taken to determine whether the effects of a gene are realized through modification of the module to which it contributes, or are realized through effects of that single gene functioning independently of its contribution to modular function. One approach would be to show whether or not the effects of multiple, independent mutations or single gene knock-outs that each eliminated modular function also each had an identical effect on the phenotype. If not, the gene in question would be having an ancillary effect on the phenotype. Complexity in connecting genotypes and phenotypes presents complications that are tedious to unravel. However, experimental conclusions will only be as robust as our willingness to apply rigorous standards in interpreting how genetic variation determines phenotypic variation.

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Supplementary data

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