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Evolutionary and functional genetics of insect diapause: a call for greater integration

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Diapause in response to seasonality is an important model for rapid evolutionary adaptation that is highly genetically variable, and experiences strong natural selection. Forward genetic methods using various genomic and transcriptomic approaches have begun to characterize the genetic architecture and candidate genes underlying diapause evolution. Largely in parallel, reverse genetic studies have identified functional roles for candidate genes that may or may not be genetically variable. We illustrate the disconnect between the evolutionary and physiological literature using a suite of studies of the role of the circadian clock in diapause regulation. These extensive studies in two different disciplines provide excellent opportunities for integration, which should facilitate rapid progress in understanding both the regulation and evolution of diapause.

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Introduction

Diapause, a form of dormancy in insects and other arthropods, is an adaptive and plastic phenotype that allows insects to persist in seasonally variable environments. Insects enter diapause in advance of unfavorable conditions and in response to predictive environmental cues (Box 1). Because it allows insects to persist and adapt to new environments, diapause has been a powerful model for understanding evolution by natural selection [1]. Moreover, natural populations often harbor ample genetic variation affecting both the capacity for and the timing of diapause (Box 1; [2,3]). This combination of strong selection and segregating genetic variation allows diapause to rapidly evolve over contemporary timescales, including in response to changing climates [4•], developing agricultural practices [5], and during biological invasions and range expansions [6]. Thus, genetics, selection and evolution of diapause inform several basic and applied topics, including the genetic architecture of rapid adaptation, responses to climate change, evolutionary physiology, ecological genetics, and opportunities to uncover novel targets for pest control (Figure 1).

Studying diapause has provided many important insights into adaptation to variable climates and how physiological plasticity is regulated in animals. Recently, rapid advances in '-omics' technologies have led to exciting progress in understanding the genetic and physiological mechanisms of diapause regulation and evolution. Previous reviews discuss the physiological stages of diapause progression [7], the hormonal regulation of diapause [8], epigenetic regulation of diapause [9], and diapause energetics [10]. Here, we focus on recent studies of genetic variation in diapause, emphasizing the progress achieved by '-omics' approaches. We also point out a continuing disconnect between forward genetic methods and reverse, functional genetics, using the role of the circadian clock in regulating diapause as an example. Combining these tools provides a powerful approach to better understand how diapause evolves, and to better leverage genetic variation to elucidate how this complex phenotype is regulated.

Genetic variation for diapause phenotypes over space and time

A rich history of studying genetic differentiation across latitudinal gradients (i.e. 'clines') provides an expansive view of how insects adapt to spatial environmental heterogeneity [11,12]. Clines in diapause incidence and/or annual timing (Box 1) have been described in hundreds of species and are among the most robust biogeographic trends in animals [3,13]. These clines are formed as geographic populations adapt to local conditions along seasonal gradients. Geographic variation in diapause phenotypes (Box 1) has often been leveraged to infer genetic architecture by crossing laboratory strains derived from different geographic populations [14,15]. Early studies often identified simple, nearly Mendelian factors underlying diapause variation. The advent of DNA sequencing and genetic tools enabled finer, molecular genetic

Box 1 Diapause concepts and terms

Diapause: A physiologically dynamic and hormonally controlled state of decelerated or arrested morphological development that allows insects to survive unfavorable conditions. Diapause is typically induced/terminated by environmental stimuli (e.g. photoperiod or temperature), though some diapause responses may appear functionally obligate in the field (Figure B1).

***Phases of diapause*:** Different eco-physiological states through which diapausing organisms progress (Figure B1). They are typically described as:

1 **Initiation/Induction:** The period before an insect enters diapause, characterized by some combination (but not always all) of:

- a *assessing environmental conditions* which may be mediated through measuring daylength via the circadian clock
- b *regulating energetic resources* (increased feeding, decreased insulin signaling/PI3K and upregulating fatty acid synthesis)
- c *upregulating stress responses* (e.g. HSPs [pictured], FOXO activity, cryoprotectants, immune responses)
- d *regulating hormone levels*, for example, prothoracicotropic hormone (PTTH; larval and pupal diapause), Ecdysone (20HE; pupal diapause) or Juvenile Hormone (JH; adult diapause)
- e *seeking out protected overwintering sites*

2 **Maintenance:** The period during which the organism is unresponsive to changes in the environment. This usually characterized by cell cycle arrest and decreases in transcription, cellular respiration and metabolism, allowing organisms to conserve energy reserves.

3 **Termination:** The period during which the organism becomes competent to resume normal growth, development and activity in response to favorable environmental conditions. This is generally characterized by increases in transcription, cellular respiration, metabolism, and hormonal signaling.

***Population-level diapause metrics*:** Genetic, physiological and ecological studies generally focus on the photoperiodic initiation (more common) or termination (less common) of diapause because it is closely tied to seasonal timing and phenology. Two common metrics include:

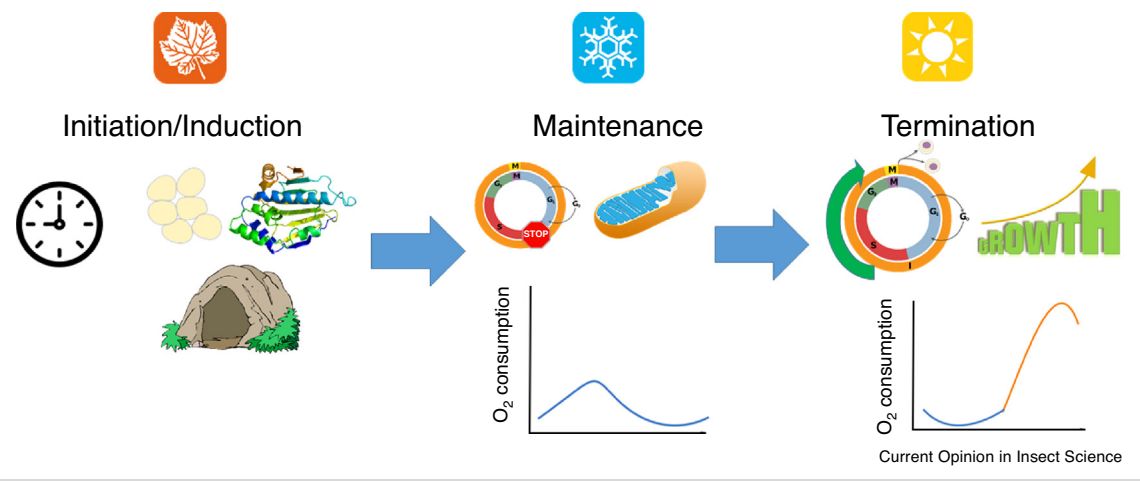
1 **Diapause incidence:** The proportion of individuals that enter diapause under unambiguous, diapause-inducing conditions (e.g. short days and/or low temperatures).

2 **Diapause timing**: The seasonal timing of diapause initiation or termination.

- a Timing is also inferred by measuring **Critical photoperiod (CPP)** in insects with photoperiodic diapause: The number of hours of light in a 24 h Light/Dark cycle that will cause 50% of the population to enter or terminate diapause (may be inadequate in some cases, see Ref. [54])

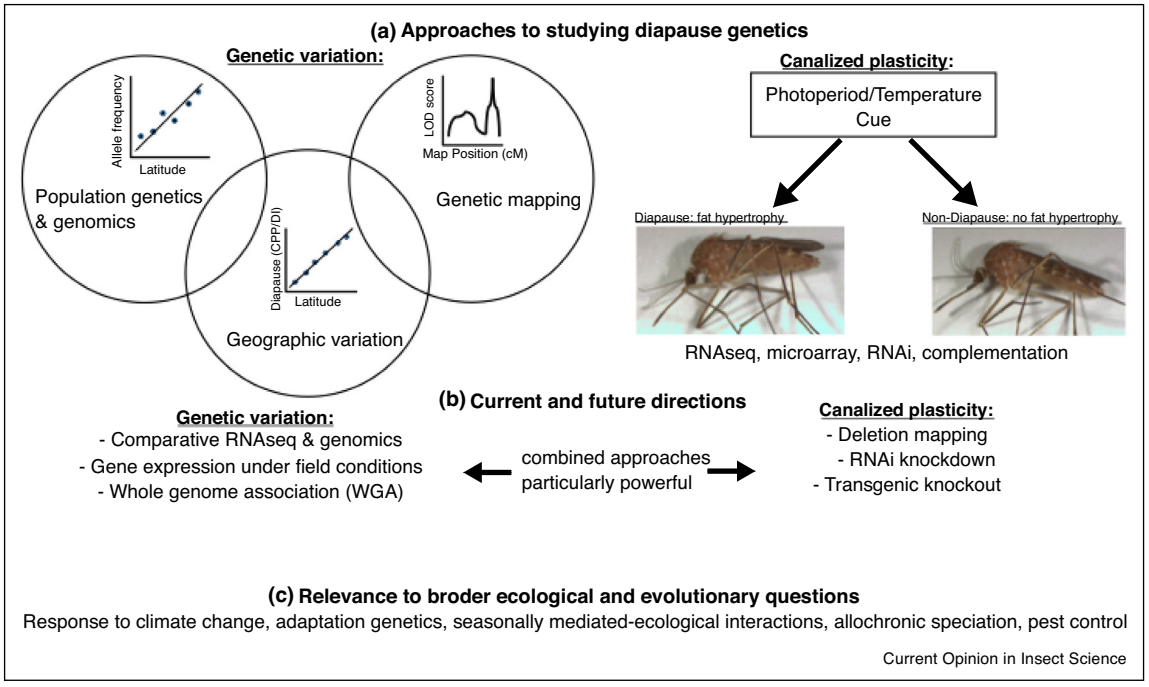
Both diapause incidence and timing vary across latitudinal and altitudinal clines, for example, CPP and diapause incidence are typically positively correlated with latitude and altitude.

Figure B1



Phases of diapause development. In temperate environments diapause initiation, maintenance, and termination typically take place in the fall, winter, and spring, respectively.

Figure 1



Overview of experimental approaches investigating diapause genetics and evolution **(a)**, potential future directions **(b)**, and relevance of diapause genetics and evolution to broader ecological and evolutionary questions **(c)**. Panel (a) highlights many ways, in which genetic variation and plasticity are evaluated. Studies analyze *genetic variation* in diapause by first documenting geographic variation in diapause phenotypes. Crossing populations with divergent phenotypes can then allow researchers to identify the genetic variants that underlie the observed phenotypic variation. Additionally, population genetics & genomics can be used to identify alleles that show coincident patterns of geographic variation. Alternatively, many studies explore the genetic regulation of *canalized plasticity* in the diapause response by exposing experimental cohorts of a single population to diapause-inducing and diapause-averting cues, and measuring differences in gene expression (RNAseq/microarrays) or determining how manipulating the level of a transcript of interest (RNAi) or the genetic background (complementation) influences the diapause response. Panel (b) highlights potential methods that are or can be used to identify genetic factors affecting diapause among and within populations, with the suggestion that combining approaches provides a particularly strong basis for identifying causal genetic factors. Panel (c) highlights the potential of understanding the regulation and evolution of diapause to inform research relating to several critical biological topics.

dissection, particularly for a few well-studied and experimentally tractable organisms (Table 1). For example, Williams *et al.* [16] crossed geographically derived lines of *Drosophila melanogaster* and identified a locus of major effect for diapause incidence. Deletion mapping further resolved the locus to variants in the insulin-regulated phosphatidylinositol 3-kinase (PI3-kinase) gene, *Dp110*, which also contributed to diapause differences among geographically disparate populations. Subsequent tests for associations between the variants and gene expression were equivocal, but the identification of *Dp110* was exciting because it supported years of comparative endocrinology suggesting a role for insulin signaling in diapause. Paaby *et al.* [17] found similar clines in alleles of the insulin-like receptor (*InR*) among *D. melanogaster* populations from both North America and Australia, though alleles were only indirectly related to diapause.

Schmidt *et al.* [18] also leveraged naturally segregating geographic variation, performing Quantitative Trait Locus (QTL) analysis and fine-mapping by crossing lines of *D. melanogaster* derived from geographic populations that differed in diapause incidence. Their experiments identified a locus of major effect, *couch potato* (*cpo*), which encodes an RNA binding protein that is highly expressed in the ring gland, the primary endocrine tissue of *D. melanogaster*. Polymorphisms at multiple SNPs were correlated with latitude, which also predicts diapause incidence in North American *D. melanogaster*. Furthermore, polymorphisms in *cpo* also change seasonally in synchrony with diapause expression [19]. Thus, the frequency of *cpo* polymorphisms is associated with diapause in both space (clinal variation) and time (seasonal variation). Higher levels of *cpo* expression are also associated with diapause maintenance in the Northern house mosquito, *Culex pipiens*, though this is the opposite relationship to that observed in *D. melanogaster* [20].

Associations between diapause and polymorphisms in genes involved in the circadian clock (Figure 2) have

Table 1

List of studies by species that apply various approaches that fall under the umbrellas of forward genetic approaches (unbiased screens for genetic variation) and reverse genetic or targeted approaches (focusing on a candidate gene or genes). Organisms studied using four or more approaches are listed in bold. With the exception of *D. melanogaster* and *C. pipiens*, most species have been studied using only one or two approaches. Moreover, single-species transcriptomics has been the most liberally applied approach

Class of Approach	Forward Genetic (untargeted)					Reverse Genetic or targeted	
Approach	Genetic Mapping (line crossing)	Genetic Association (outbred)	Single-Population Transcriptomics	Comparative Transcriptomics	Regulation Genomics	Manipulative Functional Genetics	Target Gene Variation
Questions/Goals	candidate genes; genetic architecture	candidate genes; genetic architecture	Transcriptional basis	Genetic variation in transcription	regulatory regions or molecules	target gene functional roles	targeted associations with phenotype or geography
Study organism	***D. melanogaster*** ***C. pipiens*** *O. nubilalis* *W. smithii*	***D. melanogaster*** *R. pomonella* *P. aegeria*	***D. melanogaster*** ***C. pipiens*** *R. pomonella* *Ae. albopictus* *B. minax* *D. antiqua* *S. crassipalis* *D. montana* *C. costata* *M. rotunda* *A. gifuensis* *D. antiqua* *B. mori* *T. diversipes* *H. cunea*	*R. pomonella* *O. nubilalis* *W. smithii*	***C. pipiens*** *S. crassipalpis*	***D. melanogaster*** ***C. pipiens*** *C. costata* *S. crassipalpis* *R. pedestris* *M. siamensis*	***D. melanogaster*** *O. nubilalis* *N. vitripennis*
References[a]	[1–3,4▪]	[5–7]	[8–22]	[23–25]	[26▪▪,27]	[28–32]	[33–35]

[a] Reference numbers refer to references listed in Supplemental Table S1, not to references in the main text.

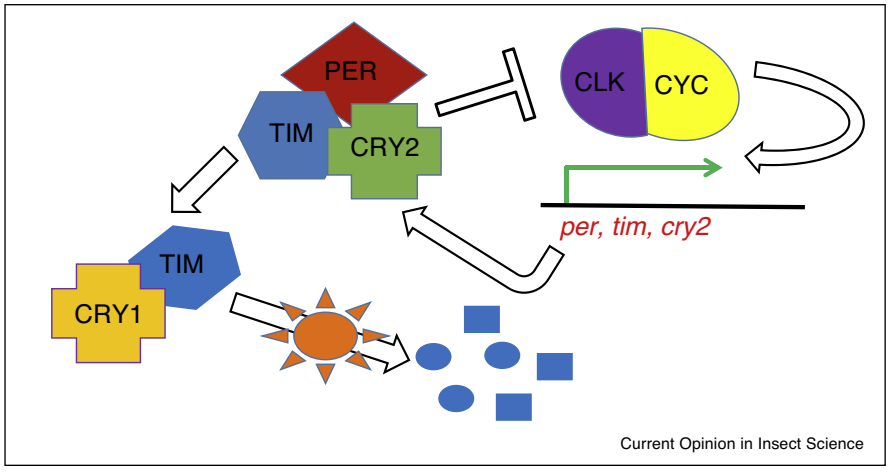
also been uncovered using both targeted and untargeted approaches. Again studying different populations of *D. melanogaster*, Tauber *et al.* [21] identified genetic variants of *timeless* in isofemale lines varying in diapause incidence. They identified a recently derived allele (*ls-tim*) encoding an additional 23 N-terminal amino acids relative to the ancestral (*s-tim*) allele. The derived *ls-tim* allele has a weaker physical interaction with the circadian light receptor Cryptochrome1 (CRY1) than the ancestral *timeless* allele (*s-tim*). Thus, the *ls-tim* allele is predicted to attenuate photosensitivy and promote entry into diapause even under long photoperiods [22]. The allele exhibits a latitudinal cline across North America coincident with the cline in diapause incidence [23,24]. Surprisingly, in Europe, the *ls-tim* allele frequency decreases with increasing latitude, but this is likely due to the recent spread of the allele from Italy to Scandinavia [25]. Other taxa exhibit geographic variation in *tim* sequence polymorphism [26••] and expression levels [27]. Furthermore, polymorphism in another core circadian clock gene, *period*, also varies with latitude and is associated with diapause variation in *D. melanogaster* [28], the European corn borer [29], the parasitic wasp *Nasonia vitripennis* [30], and the speckled wood butterfly, *Pararge aegeria* [26••]. Thus, studies of geographic variation provide substantial evidence for a link between clock gene polymorphisms and diapause (see 'The role of the circadian clock' section below).

Quantitative trait locus (QTL) analyses of crosses between seasonal populations of the European corn borer, *Ostrinia nubilalis* [31], geographic populations of the pitcher plant mosquito, *Wyeomyia smithii* [32], and members of the *C. pipiens* complex [33] also identified loci of major effect on diapause termination and diapause incidence. These results suggest that major effect loci may often segregate in natural populations. However, QTL analyses are typically unable to detect and quantify variation accounted for by loci of small effect. Furthermore, crosses between isogenic lines explore only a subset of variation segregating in natural populations, and thus may fail to detect polygenic variation. Recent studies using genome-wide association techniques suggest that segregating, polygenic variation affecting diapause may indeed be abundant (see Table 1). For example, a whole genome resequencing study in the speckled wood butterfly [26••] used Genome Wide Association (GWA) analysis to show that many loci of small effect must contribute to population differences in diapause incidence alongside several loci of major effect. A study of temperature-sensitive diapause termination in the apple maggot fly, *Rhagoletis pomonella*, also used GWA with reduced representation genome resequencing [34]. Despite finding clear evidence for heritable genetic variation, there was no evidence for loci of major effect contributing to that variation, thus supporting a polygenic model. As diapause phenotypes are highly variable in natural populations [3], it seems likely that loci of minor effect play a critical role in the evolution of diapause phenotypes, although the relative influence of loci of major effect may vary across different species.

Transcriptomic variation

Many studies in a broad range of insects and other invertebrates have compared the transcriptomes of diapausing and nondiapausing individuals, but typically in a single population of a single species (Table 1) [35,36••]. Observations of similar patterns of gene expression

Figure 2



The structure of the insect circadian clock in mosquitoes and other insects. The positive transcription factors CLOCK (CLK) and CYCLE (CYC) regulate the expression of the core clock genes, *period* (*per*), *timeless* (*tim*) and *cryptochrome2* (*cry2*), whose respective proteins inhibit the action of CLK and CYC, thereby repressing their own transcription. The TIM protein is degraded by CRY1 in the presence of light (Adapted from Ref. [53]).

during diapause across species, first through studies on single genes [37], then through transcriptomics, led to the proposal that particular genes [38] or signaling pathways [39•] may represent a common 'genetic toolkit' for diapause due to evolutionary convergence. A meta-analysis of single-species diapause transcriptomic studies indeed revealed that a common set of transcripts (including circadian clock genes) are differentially regulated during diapause, consistent with convergent evolution rather than shared evolutionary history [35]. Mechanisms that regulate gene expression (DNA methylation, histone modifications and/or individual miRNAs) have also been implicated in diapause regulation in various insects [9], but again, most of this work focuses on single populations of diapausing and nondiapausing species, and there is limited evidence that the same mechanisms are consistently used to regulate diapause across species.

In contrast to the large number of single population studies, only three studies have compared the diapause transcriptomes of genetically distinct populations of the same species. The first compared strains of the European corn borer (*O. nubilalis*) [40••]; the second compared apple-infesting and hawthorn-infesting populations of *R. pomonella* [41]; and the third compared the diapause transcriptome of southern and northern populations of *W. smithii* [42]. The goal of all three studies was to identify transcriptional changes contributing to population-level differences in diapause timing (specifically, diapause termination), and to link those changes to genetic variants.

An important advantage of comparative transcriptomic studies is the opportunity to simultaneously examine genetic variation in transcript expression and transcript sequence. Combining transcriptome comparisons with QTL studies is also particularly powerful. For example, all three comparative transcriptome studies identified polymorphisms or gene expression differences potentially related to differences in diapause termination. In the European corn borer, 48 transcripts with either fixed amino acid differences or differential expression during diapause termination among strains mapped to a chromosomal rearrangement previously identified as a major genetic factor influencing diapause termination [40••]. Several of these genes are involved in insulin signaling and the circadian clock, which have previously been implicated in diapause regulation as discussed above. Meyers *et al.* [41] found between-population expression differences in insulin and Wnt signaling, suggesting that these pathways contribute to the early spring emergence of apple-infesting flies relative to the hawthorn-infesting flies. Finally, Emerson *et al.* [42] identified a transcript designated *WsPpdrg1* that was differentially expressed among geographic populations during diapause termination. *WsPpdrg1* maps to a major QTL affecting critical photoperiod (CPP; Box 1) and is hypothesized to be involved in photoreception or signal transduction based on its similarity to *D. melanogaster* proteins. A cautionary note relevant to all of these studies is that diapause transcriptomes may differ substantially between laboratory and ecologically realistic field conditions [36••].

The intersection of evolutionary genetics and functional studies: the role of the circadian clock

Over eighty years ago the German botanist Erwin Bünning hypothesized that the same mechanism that organisms use to measure daily (circadian) time might also be used to measure seasonal (photoperiodic) time and thereby initiate responses such as diapause [43]. With the advent of modern molecular genetics, evidence supporting a role for circadian clock genes in the evolution of photoperiodism comes from: 1) screens of geographic or population-level genetic variation, 2) gene expression assays, and 3) genetic knockdown to evaluate functional consequences. In insects, the circadian clock is composed of multiple feedback loops controlled by cycling levels of key signaling proteins and transcription factors (Figure 2). Studies of genetic variation, including those detailed above, suggest that diapause is associated with variants of the core clock genes *period* and *timeless* [21,26••]. Variation in clock gene expression has been linked to natural variation in diapause [27,40••]. Furthermore, knocking down clock gene transcripts with RNA interference (dsRNAi) also suggests a functional link between the clock and photoperiodic diapause. For example, suppressing *period* causes multiple species of insects to either delay or avert diapause [44–47].

Nevertheless, the mechanistic link between the clock and the photoperiodic timer remains unresolved. Clock genes have been linked to a range of physiological processes including metabolism and hormonal signaling (e.g., juvenile hormone in adult insects; [45,47,48]). However, techniques such as transcriptome-wide screens have not identified pathways from photoperiod perception to the generation of the diapause phenotype. Additionally, unbiased, genome-wide methods often identify variants with no experimentally determined connections to the circadian clock, for example, [18,26••,42]. Emerson *et al.* [49•] noted that it is difficult to determine whether core circadian genes influence seasonal responses via their role in the circadian clock and perception of daylength or whether they pleiotropically regulate genes outside of the clock to generate diapause phenotypes. However, in the intervening decade since that review, we have yet to determine how clock gene variants mechanistically influence diapause (but see Ref. [50••], though the photoperiodic phenotype is not diapause).

Opportunities for future progress

We suggest that greater integration among what have previously been largely parallel efforts in functional

genetics, transcriptomics, and evolutionary genomics would enable more rapid progress towards identifying the molecular regulators of diapause and the genetic basis of diapause evolution. Table 1 illustrates that with a few exceptions, most '-omics' studies of diapause have been carried out in separate species. Transcriptomics of single populations of a single species are by far the most common. These single population transcriptomic comparisons (diapause to nondiapause) have uncovered similar molecular regulators across species. However, comparative transcriptomic approaches leveraging well-characterized intraspecific variation in diapause phenotypes (e.g. [40••,41,42]) provide a stronger connection between candidate genes and diapause phenotypes.

Likewise, forward genetic methods such as GWAS or QTL analysis followed by reverse genetics (e.g. generation of null mutants or RNAi) provide more robust evidence for the roles of candidate genes in diapause regulation. The studies employing genetic screens followed by deletion mapping described above illustrate the power of these combined approaches in *D. melanogaster* [16,18]. In principle, reverse genetics (e.g. creating loss of function variants) followed by transcriptomics could also identify novel mechanisms. Genome sequencing and *de novo* assembly are increasingly accessible (e.g. [26••]), RNAi has now been implemented in many species, and transgenic approaches show great promise in non-model systems (e.g. [51]) and are now becoming the preferred approach in some well-established study species [52]. We anticipate that these advances will enable powerful integrative approaches to rapidly advance our understanding of how diapause is regulated and evolves in species with genetically variable diapause responses. These advances will contribute significantly to broader issues such as determining the genetic architecture of rapid adaptation, evolutionary responses to climate change, and the identification of novel targets for pest control (Figure 1).

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cois.2019.08.003.

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- •• of outstanding interest

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