

An amino acid polymorphism in the *couch potato* gene forms the basis for climatic adaptation in *Drosophila melanogaster*

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Diapause is the classic adaptation to seasonality in arthropods, and its expression can result in extreme lifespan extension as well as enhanced resistance to environmental challenges. Little is known about the underlying evolutionary genetic architecture of diapause in any organism. *Drosophila melanogaster* exhibits a reproductive diapause that is variable within and among populations; the incidence of diapause increases with more temperate climates and has significant pleiotropic effects on a number of life history traits. Using quantitative trait mapping, we identified the RNA-binding protein encoding gene *couch potato* (*cpo*) as a major genetic locus determining diapause phenotype in *D. melanogaster* and independently confirmed this ability to impact diapause expression through genetic complementation mapping. By sequencing this gene in samples from natural populations we demonstrated through linkage association that variation for the diapause phenotype is caused by a single Lys/Ile substitution in one of the six *cpo* transcripts. Complementation analyses confirmed that the identified amino acid variants are functionally distinct with respect to diapause expression, and the polymorphism also shows geographic variation that closely mirrors the known latitudinal cline in diapause incidence. Our results suggest that a naturally occurring amino acid polymorphism results in the variable expression of a diapause syndrome that is associated with the seasonal persistence of this model organism in temperate habitats.

cline | diapause | life history | mapping | tradeoff

Natural populations encounter environmental stresses that diminish individual fitness, and these stresses are variable across space and time. It is predicted that the resulting natural selection often leads to a situation in which no genotype has the highest fitness across all environments, and polymorphism is maintained. This concept is pervasive in arguments about the evolution of life history variation and associated genetic tradeoffs (1). However, the expected molecular polymorphism associated with life history tradeoffs and adaptation remains elusive. Although we would expect these phenomena to be universal, the complexities of genetic dissection of such variation suggest that the best opportunity to identify the genetic basis for life history variation lies in the study of the genetic models in their natural populations (2).

Drosophila melanogaster is a human commensal that has spread from areas of Sub-Saharan Africa to Europe and Asia, possibly over the last 5,000 to 16,000 years, and into the Western Hemisphere and Australia in the past several hundred years (3–5). This worldwide expansion from the tropics has required adaptation to the pronounced seasonality present in temperate habitats, and there are many examples of both single-gene polymorphism and quantitative trait variation that show geographic patterns associated with the transition from tropical to temperate climates in this species (6, 7). There also is good evidence that *D. melanogaster* overwinters at the adult stage in temperate habitats (8, 9), and that temperate populations do not merely reflect recurrent migration from more moderate climates

(10, 11). This overwintering survivorship clearly presents a variety of challenges, including the need for lifespan extension well beyond that typically measured in the laboratory, as well as increased physiological tolerance of extended exposure to sub-optimal conditions (12).

The best-studied adaptation to seasonality in insects is the expression of a diapause syndrome. This phenotype is analogous, and potentially in part homologous, to the dauer stage in *Caenorhabditis elegans*; unlike the genes and pathways underlying dauer formation, however, very little is known about the genetic basis of diapause in *Drosophila*. Although many aspects of insect diapause vary across taxa, the expression of diapause is associated with a suite of physiological changes that allow persistence during periods of stress exposure (12). Once thought absent in this tropical species, reproductive diapause occurs in *D. melanogaster* and is cued by exposure of adults to short days and low temperatures (13). The expression of this diapause is under neuroendocrine control (14) and results in lifespan extension, delayed senescence, and increased stress resistance (15). The incidence of reproductive diapause exhibits a strong latitudinal cline in eastern North American populations (16), varying from $\approx 30\%$ in southern Florida to 90% in New England. Diapause incidence also varies predictably with the season (high incidence in spring, lower in fall) in temperate orchard populations in Pennsylvania and New Jersey (17). The spatial and temporal patterns of diapause variation appear to reflect a robust series of life history tradeoffs between genotypes with a high and a low propensity to express diapause (17–19). These properties of reproductive diapause in *D. melanogaster* offer a unique opportunity to elucidate the genetic architecture of an important fitness trait with pleiotropic effects on life histories. Similarly, the global spread of a sophisticated genetic model organism presents us with a superior opportunity to study adaptation to novel environments that has emerged over a relatively short evolutionary period, possibly as recent as 2,000 generations in the New World.

Previous analyses in a set of isogenic *D. melanogaster* laboratory stocks and inbred lines had demonstrated that the genetic factors affecting diapause expression were entirely associated with the third chromosome (16). In this report, we use third-

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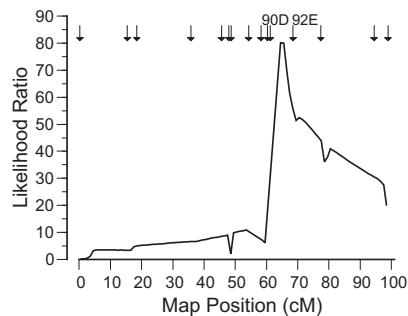


Fig. 1. Likelihood plot for the QTL analysis of reproductive diapause. The likelihood ratio, as calculated by multiple-interval mapping for ordinal traits (21) and implemented in QTL Cartographer v.2.5 (Category Trait Mapping, Forward Model), is plotted as a function of location in cM units on chromosome 3. The positions of the markers used in the analysis are indicated by arrows, and they correspond to cytological positions 61C, 64E, 65D, 68C, 75F, 83B, 85D, 87E, 89A, 90A, 90D, 92E, 94D, 98B, and 99A. The two markers that flank the identified QTL are indicated.

chromosome recombinants in a standardized genetic background, genetic complementation, and linkage association in a natural population to identify the gene responsible for diapause variation in natural populations.

Results

A set of 15 third-chromosome SNP markers was used to initially map the reproductive diapause phenotype in 250 recombinant inbred lines (RILs) generated from recombination between the parental lines *w;6326;VT46* and *w;6326;6326*. These lines were selected because the inbred line *VT46* does not exhibit diapause, whereas the isogenic line *6326* expresses diapause (16) and also forms a reference genome in other studies (20). Using these markers, multi-interval mapping (21) places a single identified quantitative trait locus (QTL) between markers at cytological band positions 90D1 and 92E8 (Fig. 1). A subset of 20 RILs possessed informative recombination events within the identified interval; the placement of an additional eight SNP markers in these lines provided a higher-resolution map (Fig. 2). This refined analysis eliminated a number of genes in the initial 248-kb region and identified a final candidate gene as *couch potato* (*cpo*).

To independently test the hypothesis that variation in diapause expression is associated with the *cpo* gene, we carried out four

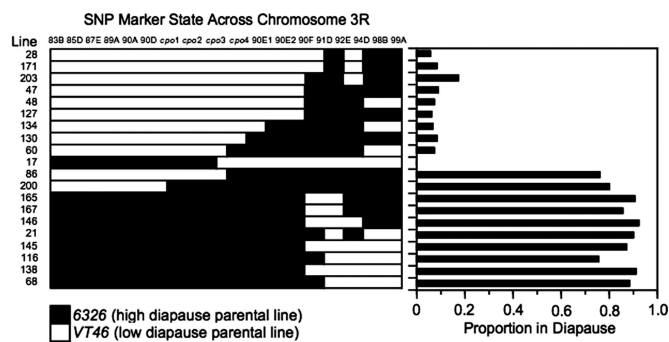


Fig. 2. SNP genotypes across the right arm of the third chromosome and diapause incidence in the 20 RILs that were recombinant in the interval to which diapause mapped. The cytological position of each SNP marker is given (Left, top) and ranged from band 83B to 99A. Four SNPs were placed in the *cpo* gene and are listed as *cpo1*–*4*. The variation in diapause phenotype clearly maps only to the interval between SNP markers *cpo3* and *cpo4*; this corresponds to the 3' end of the *cpo* locus containing all of the coding sequence in exon 5.

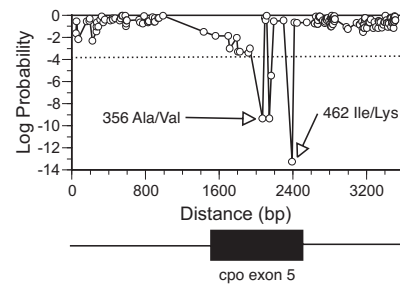


Fig. 3. The association between diapause phenotype and each of 192 identified polymorphisms in the 3.5-kb region encompassing *cpo* exon 5. Each point represents the transformed *P* value resulting from a nominal logistic regression of nucleotide state on diapause incidence in the 35 extracted third chromosomes from the DPF population. The dashed line indicates significance threshold based on Bonferroni adjustment for multiple testing. The four sites (two synonymous and two nonsynonymous substitutions) that are significantly associated with diapause phenotype span 322 bp and are in significant linkage disequilibrium with one another (*D* ranges from 0.162 to 0.238, $P < 0.0001$ for each). The amino acid polymorphism at residue 462 is present in only the smallest *cpo* transcript (*cpo*-RH).

sets of genetic complementation studies using *cpo* P element and *piggyBac* transposon-derived constructs in a standardized genetic background. These analyses used the alleles *cpo*^{BG02810} (22) and *cpo*^{P3} (the latter is a precise excision of the *P*{*w*^{+GT}} element in *cpo*^{BG02810}), as well as the homozygous viable *cpo* hypomorphs *cpo*^{v3}, *cpo*^{cp1}, and *cpo*^{cp2} (23). A second set of crosses used FLP-FRT site-specific recombination (20) of *piggyBac* elements to create duplications and deletions of both the *cpo* gene region (90C6–90E1) and the 5' flanking region outside *cpo* (90B7–90C1). These studies all confirm that genetic modifications of the *cpo* gene alone cause pronounced and repeatable effects on diapause expression [supporting information (SI) Text and Fig. S1].

Since *cpo* dosage appears to influence diapause, we measured the *cpo* transcript levels in the progenitor *VT46* (RIL line 201) and *6326* (RIL line 107) lines. The results of the RT-PCR that includes *dp110* and *Gapdh* controls showed that *6326* third instar larvae and adults possess $\approx 15\%$ of the level of *cpo* transcript as *VT46* (SI Text and Fig. S2).

We next determined whether diapause phenotype in natural populations was associated with molecular polymorphism within the *cpo* locus. The *cpo* gene spans 84 kb and encodes six discrete transcripts. The major coding region (exon 5 of the *cpo*-RA transcript) is 449 aa separated by 37.5 kb from a scattered 3' set of seven small exons encoding another 286 aa (depending on the transcript and annotation). Preliminary sequence analysis of exon 5 in a diverse set of lines identified extensive silent and amino acid polymorphism, as well as two major haplotypes. The central portion of the exon also contains several short tracts of polyglutamine repeats that vary in copy number. We began our linkage disequilibrium association analysis by examining this major exon and its variation.

We sequenced 3.5 kb spanning exon 5 from 35 third chromosomes from a single natural population (Davis Peach Farm; DPF) that were placed into the standardized genetic background (*w;6326*) used throughout this study. The DPF third-chromosome lines were then assayed for diapause expression under the standard induction conditions. The association between diapause phenotype and each of 192 polymorphic sites over the 3.5-kb region is depicted in Fig. 3. The only polymorphic sites that were significantly associated with diapause phenotype are located in the 3' end of *cpo* exon 5, and these reflect the aforementioned major haplotypes. Two of these polymorphisms are nonconservative amino acid changes. Amino acid position

cause is an isoleucine-to-lysine mutation in residue 462 that also shows a latitudinal cline consistent with that previously observed for diapause incidence. This does not rule out other genes contributing to the variation in diapause in natural populations, as these analyses were conducted in a standardized genetic background where the second and X chromosomes were isogenic. The manipulations of levels of *cpo* expression by hypomorphic alleles and gene duplications/deletions support the hypothesis that variation for diapause phenotype in *D. melanogaster* is associated with *cpo* expression level. This hypothesis was further supported by initial RT-PCR expression analyses of the parental lines used to map diapause to *cpo*. Given these results, it is interesting that the candidate site is an amino acid polymorphism in the coding region of a single transcript: the change in primary protein sequence may be of direct functional significance and/or affect some aspect of dosage. At present, there are no data to test these hypotheses.

The *couch potato* gene was first identified in a screen for genes expressed in sensory organ precursor cells during peripheral nervous development (23). It was shown subsequently to produce a nuclear protein and encodes an RNA-binding domain that is expressed in the peripheral and central nervous system of embryos, larvae, and adults, and in such tissues as the midgut, glia, and salivary glands (28). Also, *cpo* is highly expressed in the ring gland (29), the primary endocrine structure in *Drosophila*; this is particularly interesting because diapause is under neuroendocrine control. We also identified a series of *cpo* ecdysone response elements, suggesting that the effects of *cpo* on diapause in *D. melanogaster* may be mediated by ecdysteroids (30). Loss-of-function *cpo* mutations are lethal, and partial loss-of-function mutations generate a variety of behavioral phenotypes (23) and neurological abnormalities (31). The three predicted ORFs contain a nuclear localization sequence, polyglutamine OPA repeat regions, and an RRM-RNA recognition motif in five of the six transcripts (28). We see that the RRM region is very highly conserved across the 12 sequenced *Drosophila* genomes, but the OPA repeats show extensive copy number variation across taxa. Of particular note is that this alternate splicing predicts that the Ile/Lys amino acid polymorphism in residue 462 lies in the only protein lacking the RNA-binding domain. Our RT-PCR was not designed to target this transcript specifically.

Two recent studies have also investigated the genetic architecture for the reproductive diapause trait in *D. melanogaster*. Williams *et al.* (32) linked natural variation in reproductive diapause to the insulin-regulated phosphatidylinositol 3-kinase gene, *dp110*, also found close to *cpo* on the third chromosome at band 92F3. This association with diapause was further supported by manipulations of *dp110* using transgene constructions. However, no naturally occurring amino acid sequence variation was observed in the *dp110* gene, and no significant differences in *dp110* expression were detected between the high- and low-diapause lines. In *C. elegans* the homologue of *dp110*, *age-1*, is associated with dauer formation, and this concurrence suggests a common role for this gene in countering environmental stress across species. The identification of *dp110* as a diapause gene also suggests that aspects of diapause in *Drosophila* may be regulated by insulin signaling, and that *C. elegans* dauer and *D. melanogaster* diapause may be more than analogous phenotypes (33). Insulin signaling also has been shown to affect diapause expression in the mosquito *Culex pipiens* (34).

Diapause induction was also studied in association with allelic variation in the gene *timeless* (*tim*) (35), which encodes a light-responsive component of the circadian clock (36). As diapause expression is dependent on photoperiod, the authors hypothesized that this gene might impact reproductive diapause. In particular, a newly derived allele, *ls-tim*, increases in frequency across Europe, raising a possible association between temperate

habitats, diapause incidence, and *tim* allele frequencies. Tauber *et al.* (36) showed that in three populations there was a significant relationship between the proportion expressing diapause, the testing photoperiod, and homozygous genotypes for the *timeless* alleles *s-tim* and *ls-tim*. It will be interesting to determine whether a similar association exists for the *timeless* alleles and diapause in North American populations, and in particular whether there is a latitudinal cline, as would be predicted.

In *D. melanogaster* the genetic variance associated with reproductive diapause has been shown to have profound pleiotropic effects on a number of other fitness-related phenotypes. These include lifespan, rates of senescence, fecundity profiles, development time, lipid content, and resistance to a variety of stressors (16–19). The absence of diapause induction in *Drosophila simulans* and African populations of *D. melanogaster* (19) suggests that this trait is of recent evolutionary origin (14) or is very rare in these populations. Our analysis identified a single gene and single nucleotide polymorphism that explains the observed variance in diapause expression in natural populations. However, it is likely that the diapause trait is polygenic in *D. melanogaster*, and that more genes will be discovered that modify its expression and variation. Nevertheless, the combined quantitative mapping analysis, complementation studies, detailed evaluation of molecular variation, and the predicted association of geographic variation are compelling in singling out *cpo*. A number of important questions remain. These concern the function of *cpo*, and in particular that of the splicing product *cpo*-RH that replaces the putative RNA-binding domain with a highly basic lysine/arginine rich terminus of 41 aa. Whether this product in turn indirectly impacts many downstream genes or acts more directly remains to be deciphered to explain the many pleiotropic effects that variation in this gene has on life history traits.

Materials and Methods

Stocks. All lines and *cpo* alleles were placed into a common genetic background of *w;6326* using marker-assisted introgression (37). The complementation analyses used *cpo*^{BG02810} (22) and *cpo*^{P3} (a precise excision of the *P*(*w*⁺^{G7}) element in *cpo*^{BG02810}). Mobilization of the *P* element used standard crosses to a male stock carrying a transposase source; excisions were recovered in males, extracted, and the background replaced. Excision was confirmed by PCR fragment analysis and sequencing of the gene region. The other *cpo* hypomorphs used (*cpo*^{v3}, *cpo*^{op1}, and *cpo*^{op2}) have been described (23). Line *w;6326;VT46* (low-diapause parental genotype) is derived from an inbred line, *VT46*, collected in 1997 from Whiting, Vt (16). Line *w;6326;6326* (high-diapause parental genotype) is a derivative of Bloomington line 6326 with the white-marked chromosome from Bloomington stock 2475.

To create site-specific duplications and deletions across the *cpo* gene region, *piggyBac* FLP-FRT-facilitated recombination (20) was carried out between insertions *PBac*(*WH*)*CG7357*[f00521] and *PBac*(*WH*)*CG7785*[f06154]. More than 100 third recombinant chromosome lines were recovered over the *TM3* balancer chromosome, screened, and characterized. The manipulation of the *cpo* gene region resulted in a 214-kb duplication (designated *cpo*^{3.20}) and deletion (*cpo*^{3.42}) that span polytene bands 90C6 to 90E1. This region also covers four other genes (*CG31246*, *tinc*, *Rim*, and *Dnase2*). The selected gene deletion (*cpo*^{3.42}) is homozygous, lethal, and is clearly visible in polytene preparations. The selected gene duplication (*cpo*^{3.20}) was confirmed by PCR fragment analysis (20). Polytene chromosome preparations showed *cpo*^{3.20} as a duplication. Duplications and deletions of the 5' region immediately flanking *cpo* were created by *piggyBac* FLP-FRT-facilitated recombination between insertions *PBac*(*WH*)*CG14325*[f03448] and *PBac*(*WH*)*CG31249*[f07289]. This region covers 58.14 kb and includes eight genes (*alt*, *ald*, *CG31360*, *CG31249*, *CG31251*, *CG7655*, *CG7523*, and *CG14322*) and 10 tRNAs. Allele *cpo*^{1.36} was confirmed by PCR and fragment analysis as a genomic duplication and was homozygous viable; allele *cpo*^{1.45} was confirmed as a deletion that was homozygous lethal. All third-chromosome lines were placed into the common *w;6326* genetic background.

Mapping. To map diapause QTL, a single F₁ female from a cross between the high-diapause (*w;6326;6326*) and low-diapause (*w;6326;VT46*) parental lines was mated to *w;6326;TM3/Dr* males, and more than 250 recombinant male

progeny were recovered. Each *w;6326;TM3/+RIL* male was mated to the *w;6326;TM3/Dr* balancer line to directly establish RILs for the third chromosome. All of the lines had identical X and second chromosomes. Using restriction site polymorphisms designed from Hoskins *et al.* (38) and from direct sequencing, 15 SNP sites were used initially to map 275 recombination events in 201 homozygous fertile lines. All lines were phenotyped for diapause, and we recovered 112 high-diapause and 79 low-diapause RILs. Each RIL was discretely partitioned into a high- or low-diapause class (see methods below). Thus, the phenotype data were binary in nature and were analyzed by multiple-interval mapping for ordinal traits (21). This method is a maximum likelihood-based approach that uses multiple marker intervals to determine significance for models that contain variable numbers of putative QTLs. Data were analyzed in Windows QTL Cartographer 2.5. Subsequently, an additional eight SNP markers were designed within the interval between cytological bands 90D1 and 92E8 by direct sequencing of parental lines.

Diapause Phenotyping. The diapause induction phenotype of the RILs was tested in the homozygous condition ($+_{RIL}/+_{RIL}$), as well as over the *TM3* balancer ($+_{RIL}/TM3$), which expresses a low-diapause phenotype (16). These results indicated codominance of high- and low-diapause alleles: $+_{HD}/+_{HD}$ flies were nonvitellogenic, but $+_{HD}/TM3$ flies contained stage 8 oocytes and a mean of 1.2 stage 14 oocytes per set of ovaries. In contrast, $+_{LD}/+_{LD}$ flies, as well as $+_{LD}/TM3$ flies, were strongly vitellogenic, with an average of 14.3 stage 14 oocytes per ovary set. For purposes of mapping analysis, all lines were scored in the homozygous state. All lines and crosses that were phenotyped were maintained at low density in vial cultures on standard cornmeal-molasses medium at 25°C, 12L:12D. Females were collected within 2 h of eclosion from replicate vial cultures and placed at 11°C, 10L:14D, in Percival I36V incubators. These females were dissected 4 weeks later, and the developmental status of the ovaries was assessed according to King (39). A female was scored as diapausing if egg development was arrested before vitellogen-

esis (before stage 8); a female was scored as nondiapausing if vitellogenin was observed in either ovary (stage 8 or later). At least 100 females were scored from 10 replicate cultures for each RIL and line cross. A bimodal distribution was observed: the incidence of diapause in a given RIL was either high (>70%) or low (<25%), with no intermediate frequencies observed. Thus, the trait was treated as binary and each RIL scored as a high- or low-diapause genotype.

Allele Frequency Clines. Eleven populations from the US East Coast were included in this study. Ten were collected by Brian Verrelli in 1997 and have been described previously (7). The DPF population was collected in 2005 in Mount Sinai, NY, by Thomas Merritt. Isofemale lines were immediately established in the field from these populations, and the third chromosomes were extracted using a *TM3/Dr* stock. A subset ($n = 46$ for DPF, $n = 48$ for all other populations) of homozygous, viable, third-chromosome lines was used in the restriction fragment length polymorphism assay. The primer pairs and restriction enzymes are: exon 1 reference SNP, TspRI: 5'-GTCAAAGCGGGGA-AAATATAGC-3' and 5'-AAATGTGTGGTAAAACCTCTGCG-3'; exon 5 SNPs, AfeI: 5'-ACAGCAACACCAAGTGCAGGAG-3' and 5'-TCCATGCTCTGCGAAAGTCC-3', and BsiEI (residue 356 Ala/Val): 5'-ACAGCAACACCAAGTGCAGGAG-3' and 5'-TCCATGCTCTGCGAAAGTCC-3'; and both exon 6 SNPs, Ddel: 5'-CGCTCAAAGTAAAGCTGCG-3' and 5'-CTCACCGATGCAGTTTGGC-3'.

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