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Research article

The Genetic Basis of Natural Variation in Drosophila (Diptera: Drosophilidae) Virgin Egg Retention

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Abstract

Drosophila melanogaster is able to thrive in harsh northern climates through adaptations in life-history traits and physiological mechanisms that allow for survival through the winter. We examined the genetic basis of natural variation in one such trait, female virgin egg retention, which was previously shown to vary clinally and seasonally. To further our understanding of the genetic basis and evolution of virgin egg retention, we performed a genome-wide association study (GWAS) using the previously sequenced Drosophila Genetic Reference Panel (DGRP) mapping population. We found 29 single nucleotide polymorphisms (SNPs) associated with virgin egg retention and assayed 6 available mutant lines, each harboring a mutation in a candidate gene, for effects on egg retention time. We found that four out of the six mutant lines had defects in egg retention time as compared with the respective controls: *mun, T48, Mes-4,* and *Klp67A*. Surprisingly, none of these genes has a recognized role in ovulation control, but three of the four genes have known effects on fertility or have high expression in the ovaries. We also found that the SNP set associated with egg retention time was enriched for clinal SNPs. The majority of clinal SNPs had alleles associated with longer egg retention present at higher frequencies in higher latitudes. Our results support previous studies that show higher frequency of long retention times at higher latitude, providing evidence for the adaptive value of virgin egg-retention.

Key words: Drosophila Genetics Reference Panel, virgin egg retention, GWAS, cline

Various aspects of temperate environments challenge ectotherm populations, and many species exhibit patterns of local adaptation in direct response to spatial variation in such selection pressures. Spatially varying selection pressures often generate patterns of clinal variation in a variety of life-history traits, such as reproductive diapause, fecundity, longevity, and stress tolerance (Boulétreau-Merle et al. 1982; Mitrovski and Hoffmann 2001; Schmidt et al. 2005; Umina et al. 2005; Arthur et al. 2008; Schmidt and Paaby 2008). In addition to latitudinal clines, life history traits adaptively evolve over seasonal time scales in direct or indirect response to seasonal fluctuations in climate (Boulétreau-Merle et al. 1987, 1992; Schmidt and Conde 2006; Behrman et al. 2015). In the current study, we focus on *Drosophila melanogaster* as a model species for climatic adaptation because of the large span of its habitat and wealth of genetic tools that can be used to dissect genetic variation in life-history traits.

Virgin egg retention is a life-history trait that varies clinally, but has not yet been investigated with fine-scale mapping of natural genetic variation. The egg retention time of *Drosophila melanogaster* virgin females, or delay in the laying of unfertilized eggs, varies both with seasons and along latitudinal gradients (Boulétreau-Merle 1990; Boulétreau-Merle et al., 1992). Genotypes displaying longer egg retention in French *D. melanogaster* populations are more prevalent in spring and autumn and at higher latitudes (Boulétreau-Merle et al. 1992). Furthermore, long egg retention genotypes are associated with higher viability of pupa, longer life span even upon insemination, and higher fecundity at low winter temperatures than short retention genotypes (Boulétreau-Merle et al. 1992; Boulétreau-Merle and Fouillet 2002). A possible explanation is that a long egg retention phenotype minimizes pointless loss of reproductive potential, as both virgin and mated long retention females retain eggs longer at low temperature than short retention females (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002). This is evidence that longer retention has adaptive value for overwintering survival, as the capacity to restart the population once spring arrives would be mostly dependent on females

that were fertilized in autumn (Bouletreau-Merle et al. 1989; Santiago et al. 1989). Virgin egg retention is genetically determined, with major genetic components on the third chromosome and minor modifiers on the X chromosome, although significant interactions between developmental temperature and chromosomal origin implicate genetic components on the first and second chromosome as well (Bouletreau-Merle et al. 1989; 1998). Until now, naturally segregating genetic variants responsible for this trait have not been mapped at a finer scale.

Here, we investigate the genes and genetic variants underpinning natural variation in virgin egg retention in North American *D. melanogaster* through a genome-wide association study using inbred lines. Subsequent investigation of a set of candidate genes was performed through experiments using null mutants, gene expression analysis, correlation with other life-history traits, and enrichment analyses for clinality and seasonality. In general, we were able to verify that a majority of tested candidate genes affect virgin egg retention time and that some of the natural polymorphisms underlying virgin egg retention time may adaptively evolve in response to spatially varying selection pressures.

Methods

Drosophila Stocks and Rearing Conditions

We employed 90 lines from the Drosophila Genetic Reference Panel (DGRP; Mackay et al. 2012) for our initial association mapping study; a subset of the full 205 DGRP lines were used for logistic reasons. We note that the limited number of lines used restricts our analysis to the identification of common variants of large effect. Flies were reared on media containing 6.25% unsulfured molasses, 7.8% cornmeal, 3% yeast, and 1.25% agar on a 12L:12D light cycle at 25°C. Classical mutant flies for validation experiments were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN).

Screening DGRP Lines for Egg Retention Phenotype

Ninety DGRP lines were screened in three blocks of ~30 lines over the course of 3 mo, with each block taking 1 mo to screen. Ten virgin females were collected from each DGRP line and placed into individual vials for the assay. The females were kept on 2% yeast media until the first egg was laid or the fly died. The vials were kept on a 12L:12D light cycle at 25°C over the course of the assay. The vials were checked for eggs in the morning and in the afternoon by examining the media in the vial under a dissection microscope. Egg retention time was measured by recording the date of initial placement in the vial and the date of the first egg. Vials were kept for an extra 3–4 d after the first egg was deposited to check for presence of larva, which would indicate that the female had been fertilized. Vials containing larva or vials where the flies died before laying the first egg were excluded from the analysis. Overall, 71 vials were removed from the 796 vials in the screen due to fly death or fertilization of the virgin female.

Genome-Wide Association Mapping for Egg Retention Phenotype

Random effect terms for each DGRP line were calculated using a generalized linear mixed effects model in R, using the LME4 package (Bates et al. 2015). The model employed was $Y_{ab} = \mu + line_a + date_b + \varepsilon_{ab}$, where Y is the age of females when eggs are first laid, $line_a$ represents the random effect of DGRP line, $date_b$ is the random effect of date of virgin female collection for the DGRP line, and ε_{ab} is the error term. We assumed a Poisson distribution of waiting times. Heritability was calculated as the variance explained by line divided by the sum of all sources of variance but for this analysis, we used the package MCMCglmm (Hadfield 2010) to calculate these variances. The values for each DGRP line were uploaded to the DGRP2 webtool (Huang et al. 2014), which performs mapping taking into account inversion and *Wolbachia* infection status. We chose a significance threshold of $P < 10^{-5}$ to identify polymorphism for further consideration. While this is a liberal threshold that may include false positives, it has become the standard for DGRP studies (Mackay et al. 2012) and we view association mapping as a means of generating hypotheses about genes involved in the natural variation for the phenotype and will functionally validate the associations below. We also note that limited number of DGRP lines tested means that our experimental design has the strongest power to detect common variants.

Functional Validation of Genes Containing Associated SNPs

Classical null mutant lines were obtained for all genes (where available) containing SNPs significant at a threshold of $P < 10^{-5}$. The mutant lines used were: Mi{ET1}Gfrl^{MB00064}, P{EPgy2}T48^{EY07311}, P{EPgy2}Mes-4^{EY01950}, Mi{ET1}stnB^{MB04192}, P{EPgy2}Acph-1^{EY19501}, and P{EP}Klp67^{AEP3516} (Supp Table 1 [online only]). Ten to fifteen virgin females were collected from each genotype (six mutants plus two controls, y^{1}w^{67c23} and w^{1118}) and placed individually in vials. Vials were checked daily for eggs and females were put on new vials every 5 d. After females laid eggs, vials were kept for an additional week to be sure that no eggs were fertilized because a mated female had been mistaken for a virgin. This design was replicated in three complete blocks for between 30 and 40 observations (individual virgin female flies) per genotype. Differences in egg retention between mutants and controls were ascertained by performing an ANOVA with genotype and block as factors and egg retention days as the response variable. Since all mutants were found on one of two backgrounds a Dunnett's test was performed after the ANOVA to determine which mutant(s) differed significantly from the control (Hothorn et al. 2008). Note that *Stoned-A*, *Mes-4*, *T48*, and their proper controls were tested in a separate set of experimental blocks than the others and are, therefore, presented separately from the other genes.

Power was calculated by assuming a Poisson distribution of egg retention times with observed means for the control and the mutant. We performed 10,000 random draws of the appropriate sample size from those distributions determined what proportion of those 10,000 draws resulted in a significant difference in egg retention time at $P < 0.05$. All analysis was done in R (R Core Team 2015).

Analysis of Enrichment of Clinal SNPs

We examined whether our top GWAS hits were enriched among sets of previously identified clinal and seasonal SNPs from samples collected along the east coast of North America (five populations spanning from Florida to Maine) or among seasons (spring and fall over three years) in a focal North American population (Bergland et al. 2014; Machado et al. 2016). A dataset of allele frequencies at 550,895 SNPs sampled from five populations along the east coast of North America was used to assess enrichment of clinal SNPs in the GWAS results (Bergland et al. 2014; Machado et al. 2016). Seven of the SNPs from our GWAS results ($P < 10^{-5}$) matched the SNPs from Bergland et al. and Machado et al. datasets (Table 1: 3L:1696552, 3L:16448208, 3R:22722373, 3R:23759237, 3R:27115805, X:3655147, X:22397621). These seven SNPs were used to assess enrichment of clinal and seasonal SNPs in our results. One thousand

Table 1. SNPs significantly associated with virgin egg retention ($P < 10^{-5}$). MAF is minor allele frequency, Del, deletion, for non-synonymous SNPs, A55G is interpreted as a mutation that causes a change from alanine to glycine in the 55th position of the protein, SNPs downstream of the gene are denoted as "Down (position)".

Chr	Pos	MAF	Effect	*P*-value	Gene	Class
X	22394286	0.384	−0.120	5.86E−07	*StnA/B*	Del (78bp—intron)
X	22385304	0.333	−0.130	5.93E−07	*StnA/B*	Intron
3L	9356607	0.180	−0.158	7.53E−07	*Klp67A*	Nonsyn (S691N)
X	22386644	0.356	−0.121	1.08E−06	*StnA/B*	Intron
2R	16443531	0.227	−0.143	1.32E−06	*lms*	Up(833)
3L	1696552	0.218	−0.148	1.38E−06	*CG7991*	Intron
3L	9356604	0.191	−0.151	1.44E−06	*Klp67A*	Nonsyn (T692S)
3L	9356609	0.171	−0.156	1.60E−06	*Klp67A*	Syn
3R	25293837	0.165	−0.157	2.24E−06	*Ptp99A*	Intron
3R	22722373	0.446	−0.105	2.56E−06	*T48*	Intron
3R	23759237	0.302	−0.124	3.95E−06	*Mes-4*	Intron
3R	16293867	0.140	−0.166	4.11E−06	*mun*	Intron
X	11867221	0.253	−0.132	4.13E−06	*cac*	Intron
X	3655147	0.144	−0.153	4.36E−06	*tlk*	Intron
3L	12152503	0.130	−0.164	4.57E−06	*CG9760*	Syn.
X	22385502	0.366	−0.117	5.89E−06	*StnA/B*	Intron
3R	24327835	0.193	−0.141	6.18E−06	*Dhc98D*	Intron
X	22397621	0.364	−0.112	6.42E−06	*StnA/B*	5' UTR
3R	16259969	0.056	−0.236	6.45E−06	*mun*	Intron
3L	9356600	0.227	−0.123	6.70E−06	*Klp67A*	Syn.
3L	16448208	0.216	−0.130	6.75E−06	*CG33158*	Intron
3R	9294054	0.057	−0.229	6.83E−06	NA	NA
3R	25816726	0.112	−0.178	7.05E−06	*Acph-1*	Down (140)
X	3655346	0.148	−0.151	7.27E−06	*tlk*	Intron
3R	23719399	0.250	−0.135	8.61E−06	*CG34353*	Intron
3R	6329823	0.229	0.137	8.66E−06	NA	NA
3R	25816740	0.122	−0.167	9.23E−06	*Acph-1*	Down (126)
X	22382756	0.345	−0.112	9.34E−06	*StnA/B*	3' UTR
3R	27115805	0.333	0.109	9.86E−06	NA	NA

sets of seven control SNPs were generated from the Bergland et al. and Machado et al. datasets by matching the GWAS SNPs by chromosome, minor allele frequency, and whether the SNP was associated with the UTR, intronic, or coding sequences of known genes vs the intergenic regions. The sets of control SNPs were used to create distributions of expected numbers of clinal (seasonal) and non-clinal (non-seasonal) SNPs, to which the observed count of clinal (seasonal) and non-clinal (non-seasonal) SNPs was compared. All analysis was done in R (R Core Team 2015).

Phenotypic Correlations With Other Traits and Gene Expression

Gene expression data using the entire DGRP mapping population for the genes used in the validation experiments was compiled from Huang et al. (2015). We first tested the association between SNP status and expression of the gene associated with that SNP using the Huang et al. dataset. For the 90 DGRP lines tested for egg retention, we calculated the Pearson correlation between gene expression for and virgin egg retention. In addition, we examined the correlation in gene expression among genes containing significantly associated SNPs.

Line means for traits from several other studies (Mackay et al. 2012; Durham et al. 2014; Unckless et al. 2015) were compiled and correlation was assessed for each trait against virgin egg retention time. All analysis was done in R (R Core Team 2015).

Results

Genome-Wide Association Mapping for Egg Retention

Initial analysis of the egg retention phenotype in the DGRP lines tested showed inter-line variability that is comparable with previous studies (Fig. 1; Bouletreau-Merle et al. 1989). In our study, heritability was estimated to be 0.596 (highest posterior density interval: 0.451–0.727). Twenty-nine of the 1,896,196 variant sites met our threshold significance cutoff of $P < 10^{-5}$ for association with virgin egg retention time. SNPs significantly associated with egg retention time were distributed across the genome and inspection of the distribution of *P*-values compared to the expected values via the QQ plot reveals that the model performed relatively well (Table 1; Supp Figs 1A and 1B [online only]). Only one SNP on the second chromosome met our significance threshold of $P < 10^{-5}$, whereas 19 and 9 met the threshold for the third and X chromosomes, respectively (Fisher's exact test (FET), $P < 0.0001$ for both comparisons). SNPs showing significant association with virgin egg retention were more likely to be in or within 1,000 bp of genes, with 26 of 29 (∼90%) of egg retention associated SNPs vs 1,509,979 of 1,896,196 (∼80%) of genome-wide SNPs, although this difference was not significant (FET, $P = 0.249$). Non-synonymous SNPs ($n = 2$) were also more likely to be significantly associated with the egg retention phenotype than synonymous SNPs ($n = 3$) when compared to the rest of the genome (48,720 non-synonymous SNPs, 167,361 synonymous SNPs), but the likelihood was not statistically significant (FET,

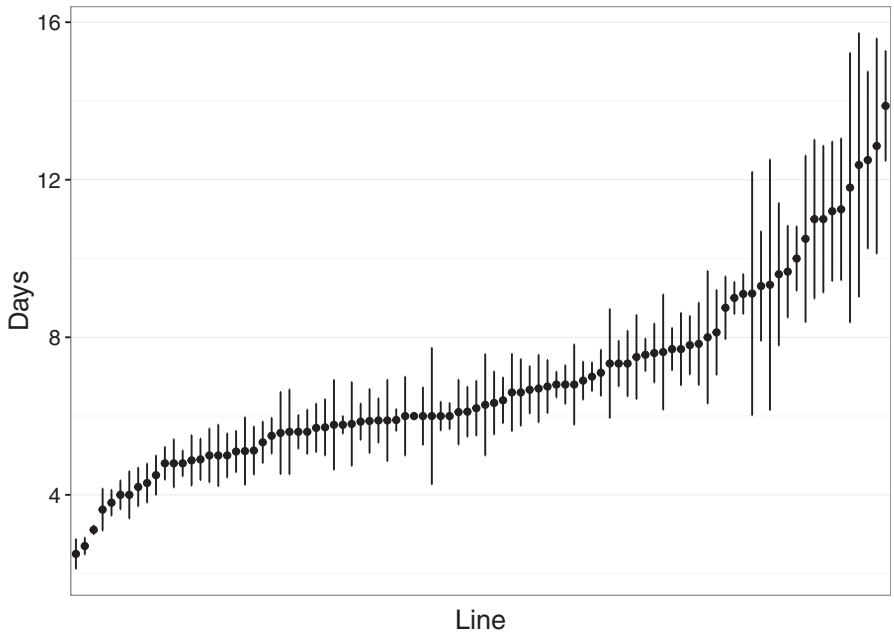

Fig. 1. Plot of mean egg retention time (± 1 SE) in ascending order for the DGRP lines screened.

$P = 0.316$). Spurious linkage disequilibrium (LD) among physically unlinked SNPs due to the small size of the mapping panel does not appear to be a problem as only tightly physically linked SNPs are in LD with each other (Supp Fig. 2 [online only]; Houle and Marquez 2015; Skelly et al. 2016).

Functional Validation of Genes Containing Associated SNPs

Four of six candidate gene mutants showed a significant difference in egg retention time compared to their controls (Supp Table 2 [online only]; Fig. 2). In all cases where differences were significant, mutant virgins retained eggs longer than their wild-type controls. Below we briefly summarize these results and putative functional roles of these genes.*mun:* An intronic SNP (3R.16259867, $P = 4.11e{-}06$) in *mun* (also known as *Gfrl*) was significantly associated with virgin egg retention in our genome-wide analysis. A second SNP (3R:16293969, $P = 6.45e{-}06$) was also significantly associated. The mutant allele for *mun* resulted in significantly longer virgin egg retention ($P < 0.0001$, mean (control) = 6.00 d, mean (*mun* mutant) = 10.28 d).

T48: An intronic SNP in *T48* was significantly associated with virgin egg retention (3R:22722373, $P = 2.56e{-}06$). The mutant allele for *T48* resulted in significantly longer virgin egg retention ($P < 0.0001$, mean (control) = 4.10 d, mean (*T48* mutant) = 6.07 d).

Mes-4: A single intronic SNP in *Mes-4* met our significance threshold (3R: 23759237, $P = 3.95e{-}06$) and the mutant allele confirmed a role of *Mes-4* in virgin egg retention ($P < 0.0001$, mean (control) = 4.10 d, mean (*Mes-4* mutant) = 6.19 d).

Klp67A: Three SNPs in *Klp67A* met our significance threshold of $P < 10^{-5}$: 3L:9356607 (non-synonymous), $P = 7.53e{-}07$; 3L:9356604 (non-synonymous), $P = 1.44e{-}06$; 3L:9356609 (synonymous), $P = 1.60e{-}06$). The mutant allele for *Klp67A* resulted in significantly longer virgin egg retention compared with the wild-type control ($P = 0.0438$, mean (control) = 6.84 d, mean (*Klp67A* mutant) = 8.15 d), although this level of significance is marginal.

Genes failing to validate with mutant lines: Two mutant genes, *StnA* and *Acph-1*, failed to show a phenotypic effect in our validation experiments. *StnA* and *StnB* are dicistronic genes. Three intronic SNPs, one in the 3′UTR, one in the 5′UTR, one 78-bp deletion in an intron were significantly associated with virgin egg retention (Table 1). Although not significant, mutants for *StnA/B* exhibited a shorter duration of egg retention ($P = 0.164$, mean (control) = 5.02 d, mean (*StnA/B* mutant) = 4.05 d). Both proteins influence neurotransmission in a calcium dependent manner (Soekmadji et al. 2012). Two SNPs (3R.25816726, $P = 7.05e{-}06$; 3R.25816740, $P = 9.22e{-}06$), 140 and 126 bp downstream of *Acph-1*, respectively, were significantly associated with virgin egg retention in our genome-wide analysis. The mutant allele for *Acph-1* led to a non-significant reduction in virgin egg retention compared to the wild-type control ($P = 0.66$, mean (control) = 6.53 d, mean *Acph-1* (mutant) = 5.82 d). *Acph-1* is an acid phosphatase primarily with no obvious connection to virgin egg retention (Tweedie et al. 2009). Note that both these analyses may have suffered from a lack of power since values are small. If we assume the means listed above and a Poisson distribution of egg retention times, then the power to detect a significant difference (assessed by simulation) at $P < 0.05$ is ~0.55 for *StnA/B* and ~0.19 for *Acph-1*. This means that if a mutation at *StnA/B* decreased virgin egg retention time, ~45% of the time the analysis would not mark it a significant difference. The same can be said for *Acph-1*, where ~81% of the time the difference in egg retention times would not be deemed significant.

Significantly associated SNPs in genes not tested: SNPs in several other genes were significantly associated with virgin egg retention but were not functionally validated due to a lack of available mutant stock, proper control, or both (Table 1).

Enrichment for clinality in significantly associated SNPs: Four SNPs out of seven significantly associated with egg retention time ($P < 10^{-5}$, Table 2) were found to have a significant change in frequency over a North American cline (Fig. 3, FDR < 0.05; Bergland et al. 2014). None of the SNPs were found to have significant change in frequency over seasons (Table 2). To determine if our set of seven SNPs was enriched for clinal SNPs, we used 1,000 sets of seven matched control SNPs to generate distributions of the number of clinal and non-clinal SNPs expected by chance alone (Fig. 4).

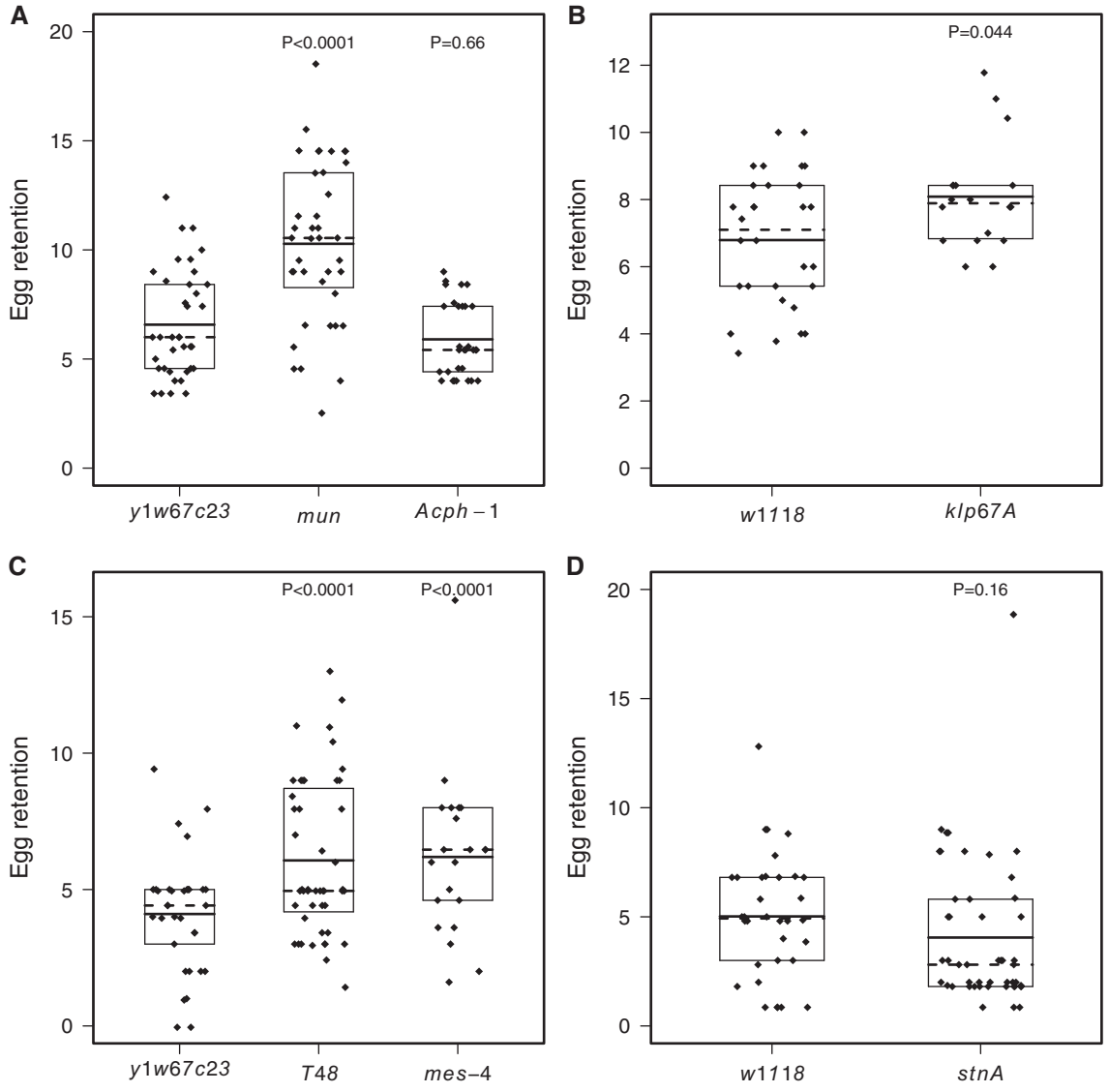

Fig. 2. Classical mutants in candidate genes show differences in egg retention times compared to their control genotypes. Each panel represents a different control (y1w67c23 or w1118) or experiment: A) first validation experiment with y1w67c23 control, B) first validation experiment with w1118 control, C) second validation experiment with y1w67c23 control, D) second validation experiment with w1118 control. Each point represents a single female corrected for block effects, boxes represent the middle 50th percentile, solid horizontal lines represent means and dashed horizontal lines represent medians. P values are given for comparison of each mutant to the control genotype using a Dunnett's Test.

Table 2. Significance of clinal and seasonal frequency changes of SNPs that are associated with egg retention ($P < 10^{-5}$). The effect (N–S) column shows the effect of each SNP on egg retention time, calculated as ½ (North Allele mean – South Allele mean). If effect (N–S) is greater than 0, then egg retention time is longer in the north vs. south.

Chr	Pos	MAF	Gene	Class	Season *q*-value	Clinal *q*-value	North allele	South allele	Effect (N–S)
3R	22722373	0.446	*T48*	Intronic	0.953	**0.025**	T	C	0.105
3R	23759237	0.302	*Mes-4*	Intronic	0.947	**0.016**	A	T	0.124
3R	27115805	0.333	NA	NA	0.788	**0.001**	C	T	0.109
3L	16448208	0.216	*CG33158*	Intronic	0.939	0.665	–	–	–
3L	1696552	0.218	*CG7991*	Intronic	0.992	0.236	–	–	–
X	3655147	0.144	*tlk*	Intronic	0.966	0.799	–	–	–
X	22397621	0.364	*StnA/B*	5' UTR	0.954	**0.044**	T	C	−0.112

The mean number of clinal SNPs expected by chance is 1.09± 0.92 (SD) and mean number of non-clinal SNPs expected by chance is 5.91 ± 0.92. Our data show an average excess of 2.91 clinal SNPs, which corresponds to an average odds ratio of 5.86. Therefore, we conclude that there is a significant enrichment of clinal SNPs in our dataset. In addition, we found that three of the four clinal SNPs (3R:

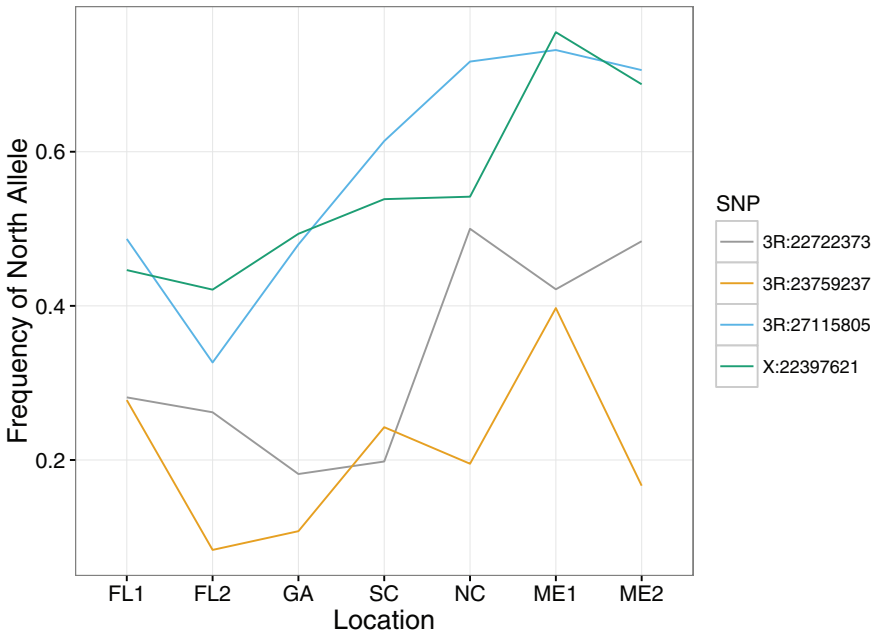

Fig. 3. Frequency of the 'north allele' along a cline for four clinal SNPs associated with variation in egg retention time. A 'north allele' is determined as the most frequent allele for a particular SNP in the northern populations. The location abbreviations correspond to the USA state from which the flies were collected and are ordered from most southern state (FL) to most northern state (ME).

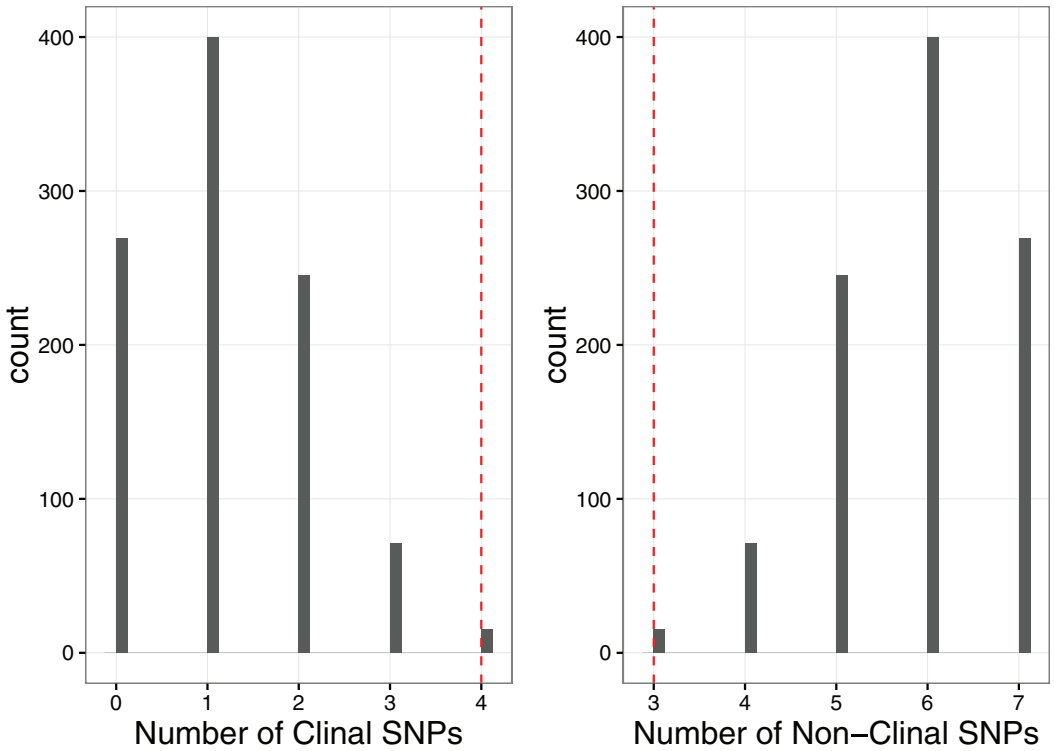

Fig. 4. SNPs significantly associated with egg retention time are enriched for clinality. The histograms present the distribution of the number of (non-) clinal SNPs from sets of control SNPs matched to our observed SNPs on the basis of chromosome and MAF. The red dashed line is the number of observed (non-) clinal SNPs from our GWAS dataset.

22722373, 3R:23759237, and 3R:27115805), of which 3R:22722373 and 3R:23759237 are in the functionally validated genes *T48* and *Mes-4*, respectively, have the allele found more frequently in the northern latitudes coincide with a longer retention time (Fig. 3; Table 2).

Correlations Among Gene Expression, SNPs and Other Traits

We examined whether variation in gene expression of genes used for validation (a) is correlated with virgin egg retention and (b) whether the state at a given SNP is associated with expression of the gene in which it falls.

Of the five genes we used for validation (no expression data was available for *StnA/B*), three genes exhibited strong positive correlation in gene expression, *Acph-1, Klp67A, and Mes-4* (Supp Fig. 3 [online only]). Pairwise correlation coefficients for these three genes ranged from 0.43 to 0.68, all of which were highly significant ($P < 0.001$). Expression of *CG34356*, on the other hand, was significantly negatively correlated with expression of the four other genes, *T48, Acph-1, Klp67A, and Mes-4* (Supp Fig. 3 [online only]). To assess the biological significance of these correlations, we calculated pairwise correlations among 1,000 randomly chosen pairs of genes from the rest of the genome. Supp Table 3 [online only] lists the percentile in pairwise correlation of each of the correlation coefficients presented in Supp Fig. 3 [online only]. Four of ten are in the top or bottom five percent of correlations and all but one are in the top or bottom twenty percent.

No genes were significantly correlated with the virgin egg retention phenotype, though *Acph-1* expression showed a moderate negative correlation. Only two downstream SNPs near *Acph-1* were significantly associated with *Acph-1* gene expression (Table 3).

Virgin egg retention was not significantly correlated with any other trait examined including measures of nutritional indices, body mass, and fecundity (Supp Table 4 [online only]). The only phenotype that came close to a significant correlation was a weak negative correlation with chill coma recovery time ($R^2 = 0.038$, $P = 0.095$).

Discussion

Virgin egg retention is associated with female overwintering success, with long retention genotype females being more likely to survive winter than short retention genotype females (Boulétreau-Merle and Fouillet 2002). Here we examined natural genetic variation in virgin egg retention using a set of inbred *Drosophila melanogaster* lines. We find genetically based phenotypic variation among lines and a genome-wide association study revealed several SNPs significantly associated with the virgin egg retention phenotype. We were able to confirm a role of four out of six tested candidate genes using classical mutants. The four validated genes were *mun, T48, Mes-4*, and *Klp67A*. We also find correlation in gene expression among these genes, though the correlation between gene expression of these genes and virgin egg retention was not significantly different from zero.

Three of the four genes validated have a role in female fertility or have high expression in ovaries. *GfrI* (*mun)* and *Klp67A* mutants show fertility defects (Gandhi et al. 2004; Kallijarvi et al. 2012). *GfrI* has been shown bind FasII, a neural cell adhesion molecule – the male and female fertility effects were hypothesized to be evolutionary ancient roles for this gene (Kallijarvi et al. 2012). *Klp67A* is a kinesin-like protein that affects spindle polymerization in mitosis and meiosis, leading to reduced fertility and also defects in embryonic cell divisions (Gandhi et al. 2004). *Mes-4* is a histone methyltransferase that is highly expressed in ovaries (Tweedie et al. 2009; Alekseyenko et al. 2014). In *C. elegans, Mes-4* is important for proper development of the germline, but no reproductive role for *Mes-4* has been identified in *Drosophila* (Fong et al. 2002). There do not seem to be direct roles of the above genes in ovulation control, but there is a significant effect on virgin egg retention. A possible explanation is that *GfrI* (*mun*) and *Klp67A* have unidentified pleiotropic mechanisms that control both fertility and virgin egg retention. No role in female reproduction or other life-history traits related to overwintering was found for *T48*. It may be that there are other unidentified pleiotropic effects of this gene.

Temperate populations of *D. melanogaster* can survive harsh winter conditions in place, whereas the *D. simulans* sister species likely recolonizes the area through migration from the south or local refugia (Boulétreau-Merle et al. 2003; Bergland et al. 2014; Machado et al. 2016). Clinal and seasonal allele changes associated with life-history traits such as fecundity, reproductive timing and potential, longevity, and reproductive diapause show that selection pressures exerted by the environment alter life-history to favor overwintering (Boulétreau-Merle et al. 1987, 1992; Mitrovski and Hoffmann 2001; Boulétreau-Merle et al. 2003; Hoffmann et al. 2003; Schmidt and Conde 2006). Virgin egg retention phenotypes not only vary clinally and seasonally (Boulétreau-Merle et al. 1992), but are also associated with other life-history traits that favor either overwintering or summertime proliferation (Boulétreau-Merle 1990; Boulétreau-Merle and Fouillet 2002). For example, long retention phenotypes have increased longevity whether virgin or mated vs. short retention phenotypes at 14°C, long enough for individuals to survive to re-establish the population under more favorable temperatures (Boulétreau-Merle et al. 1992; Boulétreau-Merle and Fouillet 2002).

In the current study, the SNPs that were significantly associated with egg retention time were enriched for clinally varying SNPs, although not for seasonally varying SNPs. All of the clinally varying SNPs were located on the third or X chromosomes, which is expected as the predicted genetic determinants of egg retention were mapped on to the 3rd and X chromosomes (Bouletreau-Merle et al. 1989). In addition, two of the clinal SNPs are located in the functionally validated *T48* and *Mes-4* genes. The clinal SNPs in these genes are not associated with changes in gene expression, though *T48* and *Mes-4* increase egg retention time if they are knocked out. Moreover, the most frequent clinal SNP alleles of the *T48* and *Mes-4* genes in the northern populations are associated with longer egg retention. This coincides with previous findings that northern populations of *D. melanogaster* have longer egg retention (Boulétreau-Merle et al. 1992). *T48* and *Mes-4* are good candidates for further study on the

Table 3. Association between significantly associated SNPs and expression of those genes.

Chr	Pos	MAF	Gene	Class	*t*-score/df	*P* value
3L	9356600	0.227	*Klp67A*	Syn.	0.667/27.65	0.510
3L	9356604	0.191	*Klp67A*	Nonsyn.	1.561/22.48	0.132
3L	9356607	0.180	*Klp67A*	Nonsyn.	1.469/20.19	0.157
3L	9356609	0.191	*Klp67A*	Syn.	1.489/18.53	0.153
3R	22722373	0.446	*T48*	Intronic	0.052/75.89	0.959
3R	23759237	0.302	*Mes-4*	Intronic	0.794/51.14	0.431
3R	25816726	0.112	*Acph-1*	Downstream	3.179/15.84	0.006*
3R	25816740	0.122	*Acph-1*	Downstream	3.385/19.43	0.003*

*Refers to $P < 0.05$.

mechanisms of how these variants affect egg retention, and perhaps other life history traits that vary clinally. In contrast to previous work (Boulétreau-Merle et al. 1992), we did not find any enrichment of seasonal SNPs, even at liberal thresholds. Association studies using more SNPs may be needed to find the seasonally varying SNPs for the virgin egg retention phenotype.

The expression levels of *Klp67A*, and *Mes-4*, genes that were validated in this study, are strongly positively correlated with each other and with *Acph-1*, a gene whose mutant did not have a significant effect on virgin egg retention, across the DGRP mapping population. The rank correlations in the expression levels of these genes fall into the top five percent of genetic pairwise correlations. *T48* has a weak positive correlation with these three genes. *Klp67A, Mes-4, Acph-1*, and *T48* are all negatively correlated with *CG34356*, with the rank correlations falling into the top or bottom twenty percent of the genetic pairwise correlations. The strong correlations in gene expression suggest that these five genes might be under similar regulatory control and that the *cis*-regulatory variants in each influence virgin egg retention. Global changes in the expression of several genes could produce the life-history changes associated with the long retention phenotype (Boulétreau-Merle et al. 1992; Boulétreau-Merle and Fouillet 2002). This is similar to the variety of changes in gene expression associated with onset of reproductive diapause (Zhao et al. 2016). Like virgin egg retention, a higher incidence of reproductive diapause is associated with predictable changes to several life history traits, such as increased life span, decreased per capita fecundity, and decreased mortality, and diapause expression is associated with overwintering survival (Schmidt and Paaby 2008; Tatar et al. 2001; Schmidt et al. 2005; Schmidt and Conde 2006). Our results indicate that virgin egg retention and reproductive diapause have different genetic bases, as the causal SNP for diapause variation did not show up in our top hits (Schmidt et al. 2008).

The correlation between egg retention and other life-history traits is not always the case, as in *D. melanogaster* populations in eastern Australia, which lack a virgin egg retention cline, but have clines for fecundity, longevity, and diapause (Mitrovski and Hoffmann 2001; Sgrò et al. 2006; Lee et al. 2011). In this case, virgin egg retention phenotype should not be predictive of overwintering success. Therefore, long virgin egg retention alone may not be vitally important for overwintering survival, but rather is often linked with a suite of other life history changes that do provide an advantage. Our results point to a weak (but non-significant) correlation with chill coma recovery time, with longer egg retention associated with short chill coma recovery time, but no significant correlations with fecundity, lifespan, or stress resistance. Previous results establishing relationships between virgin egg retention and other life-history traits were done in natural populations or recently established lines (Boulétreau-Merle et al. 1992; Boulétreau-Merle and Fouillet 2002). It is possible that the inbred nature of the DGRP lines induced a general depression in fecundity, longevity, and stress resistance that destroyed any correlation between virgin egg retention and these life-history traits.

Our study sought to find out the genes responsible for variation in virgin egg retention. Using the DGRP panel to conduct a GWAS, four genes out of the top six candidates were validated. These genes, *mun, T48, Mes-4*, and *Klp67A*, have pleiotropic effects and their expression levels are highly correlated. These genes are likely under similar regulation and operate in concert to control the physiological changes that are associated with changes in virgin egg retention. Understanding the genes associated with overwintering adaptive traits in *D. melanogaster* adds to our current knowledge of how climactic adaptation happens in cosmopolitan invertebrates, such as mosquitoes, bees, and moths (Armbruster 2016; Pitts-Singer et al. 2014; Stuckas et al. 2014). Future studies should address the roles of these genes and genetic variants in controlling virgin egg retention and overwintering survival in natural populations of *D. melanogaster.*

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Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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