

A highly pleiotropic amino acid polymorphism in the *Drosophila* insulin receptor contributes to life-history adaptation

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Finding the specific nucleotides that underlie adaptive variation is a major goal in evolutionary biology, but polygenic traits pose a challenge because the complex genotype–phenotype relationship can obscure the effects of individual alleles. However, natural selection working in large wild populations can shift allele frequencies and indicate functional regions of the genome. Previously, we showed that the two most common alleles of a complex amino acid insertion–deletion polymorphism in the *Drosophila* insulin receptor show independent, parallel clines in frequency across the North American and Australian continents. Here, we report that the cline is stable over at least a five-year period and that the polymorphism also demonstrates temporal shifts in allele frequency concurrent with seasonal change. We tested the alleles for effects on levels of insulin signaling, fecundity, development time, body size, stress tolerance, and life span. We find that the alleles are associated with predictable differences in these traits, consistent with patterns of *Drosophila* life-history variation across geography that likely reflect adaptation to the heterogeneous climatic environment. These results implicate insulin signaling as a major mediator of life-history adaptation in *Drosophila*, and suggest that life-history trade-offs can be explained by extensive pleiotropy at a single locus.

KEY WORDS: Adaptation, cline, *InR*, pleiotropy, seasonality.

Many organisms display intraspecific variation in life-history traits, including differences in reproductive timing and allocation, development, body size, stress tolerance, and life span. Alternative life-history strategies have long been hypothesized to represent adaptive responses to variable selection (Stearns 1992), but examples with known causal nucleotides are rare (Flatt and Heyland 2011). However, sequence polymorphisms have been identified recently for flowering time in *Arabidopsis thaliana* (Méndez-Vigo et al. 2013) and adaptation to freshwater environments by marine stickleback fish, which includes changes in life history as well as in physiology and morphology (Jones et al. 2012). One reason that identification of molecular targets of selection is valuable

is because it can illuminate mechanisms of multitrait correlation. For example, the gene *Catsup* in *Drosophila melanogaster* affects longevity, locomotor behavior, and bristle number, but individual polymorphisms within the locus act on the traits independently and do not show pleiotropic effects (Carbone et al. 2006). Likewise, coat color in deer mice is a multiphenotype trait associated with the *Agouti* locus, but mutations within the gene appear to have been targeted independently by selection with minimal pleiotropy (Linnen et al. 2013). These examples suggest that fitness-related traits, even those affected by the same gene, may be functionally independent such that natural populations might achieve any genetic and phenotypic combination. Alternatively,



life-history traits routinely exhibit strong genetic correlations, including negative associations between life span and reproduction and positive associations between life span and stress resistance (Reznick 1985; Stearns 1991; Partridge et al. 2005; Harshman and Zera 2007; Vermeulen and Loeschke 2007; Toivonen and Partridge 2009). These associations mirror pleiotropic effects of laboratory-derived mutations and can be difficult to break in artificial selection experiments (Partridge et al. 1999; Leroi et al. 2005; Anderson et al. 2011a; but see Khazaeli and Curtsinger 2010; Khazaeli and Curtsinger 2013). Consequently, pleiotropy, and specifically antagonistic pleiotropy, in which a genetic element encodes both positive- and negative-fitness phenotypes, remains an important component in the discussion of how life histories evolve (Williams 1957; Flatt and Heyland 2011).

Drosophila melanogaster are distributed across environments that range from temperate to tropical, and exhibit variation in life history that appears adaptive. At high latitudes, populations exhibit higher incidence of reproductive diapause, larger body size, higher cold stress tolerance, and sometimes longer life span and lower fecundity, relative to low-latitude populations (Capy et al. 1993; Mitrovski and Hoffmann 2001; De Jong and Bochdanovits 2003; Schmidt et al. 2005a; Trotta et al. 2006). There is substantial genetic variance for these traits and pervasive genetic correlations among them, indicating that selection in the local environment may act on some phenotypes but drive expression of others through trade-offs (David and Bocquet 1975; Anderson et al. 2003; De Jong and Bochdanovits 2003; Schmidt et al. 2005b; Rako et al. 2007; Schmidt and Paaby 2008). Tolerance to environmental stress may be especially important, and the ability to resist desiccation, starvation, and temperature stress correlates with climatic environment (Hoffmann and Harshman 1999; Hoffmann et al. 2001, 2005, 2007). This framework suggests a hypothetical selection regime: at high latitude, cold winters impose seasonal stress and favor genotypes that confer stress tolerance and overwintering ability; correlated traits such as larger body size, longer life span, slower development, and lower fecundity may evolve as coadapted responses to the same selection regime or by indirect selection via pleiotropy (Paaby and Schmidt 2009).

Few loci have been found to explain the observed genetic variance for *D. melanogaster* life history (De Luca et al. 2003; Carbone et al. 2006; Paaby and Schmidt 2008; Schmidt et al. 2008; Bergland et al. 2012; Remolina et al. 2012; Sgrò et al. 2013). Genes in the insulin/insulin-like growth factor signaling (IIS) pathway are good candidates because experimental manipulation of IIS mirrors the life-history trade-offs observed in natural populations (Clancy et al. 2001; Tatar et al. 2001, 2003; Partridge and Gems 2002; Giannakou and Partridge 2007; Grönke et al. 2010), and some IIS pathway members show evidence of adaptive response in the wild (Dantzer and Swanson 2012; Jovelín et al.

2014). We previously evaluated sequence variation at the *Insulin-like Receptor (InR)* in wild populations of *D. melanogaster* and observed striking reciprocal clines in the northern and southern hemispheres for the common alleles of a complex amino acid indel polymorphism (Paaby et al. 2010). The polymorphism disrupts a region of glutamine–histidine repeats in the first exon (Guirao-Rico and Aguadé 2009). In both North America and Australia, the most common allele is at low frequency in tropical and subtropical populations and increases in frequency with latitude, showing highest frequency in temperate populations; the second most common allele exhibits the inverse cline. These patterns appear to be responses to similar but independent selection pressures between the continents: fly populations in North America and Australia were founded at different times and from different source populations (Bock and Parsons 1981; David and Capy 1988), and nucleotide variants on either side of the polymorphism show neutral patterns across geography.

Initial tests showed that the two common alleles, which differ in length by two amino acids and are herein designated *InR^{short}* and *InR^{long}*, demonstrated significant effects on fitness traits. *InR^{short}*, the allele common at high latitudes, was associated with greater stress tolerance; *InR^{long}*, which is common at low latitudes, was associated with greater fecundity (Paaby et al. 2010). Here, we show that the allele frequency cline in North America persists five years later and that temporal changes in allele frequency within a single population correlate with fluctuations in the seasonal environment, which mimic the temperature and resource availability differences associated with latitudinal climate. We also find that the *InR* locus in general exhibits elevated patterns of clinality and seasonality relative to the rest of the genome. We more thoroughly tested the functional effects of the complex polymorphism by measuring levels of IIS and a spectrum of life-history phenotypes in *InR^{short}* and *InR^{long}* lines with randomized genetic backgrounds. We find that the alleles associate predictably with the tested traits, suggesting that the complex amino acid indel polymorphism at *InR* is an important target of contemporaneous selection in wild *D. melanogaster* populations, is highly pleiotropic, and contributes to observed trade-offs in *D. melanogaster* life history.

Methods

ALLELE FREQUENCY ESTIMATES

As described in Bergland et al. (2014), between 50 and 200 isofemale lines were established from six populations spanning Florida to Maine along the North American east coast (latitudes 25.5°N, 30.9°N, 33.0°N, 35.5°N, 39.9°N, 44.1°N). The population from Linvilla Orchard in Media, Pennsylvania (39.9°N, 74.4°W) was additionally sampled in the spring and fall in 2009, 2010, and 2011. Within five generations, a single male from each

line was pooled into population samples, which were sequenced on an Illumina (San Diego, CA) HiSeq2000 using 100-bp paired-end reads. Reads were aligned to the *D. melanogaster* reference genome version 5.39 using *bwa* version 0.5.9-r16 (Li and Durbin 2009). The indel polymorphism at *InR* was called using *UnifiedGenotyper* from GATK version 3.1-1 (McKenna et al. 2010), which identified multiple discrete polymorphisms within the complex indel haplotypes that we previously characterized (Paaby et al. 2010). That characterization identified many haplotypes of different lengths, two of which were common (herein *InR^{short}* and *InR^{long}*); the reference genome to which the new pooled samples were mapped has a haplotype of intermediate length (previously referred to as *InR²⁵¹*). Thus, the complex haplotypes, previously identified by their lengths, were decomposed into sets of shared or distinct alleles at discrete polymorphic sites.

We used our prior Sanger sequence data (see GenBank accessions GQ927177–GQ927244) to assign alleles of the discrete polymorphisms to the *InR^{short}* and *InR^{long}* allele classes. Here, we report those classifications (polymorphisms are denoted by their position in the *D. melanogaster* reference genome version 5.48). Three discrete polymorphisms in the pooled sequencing data discriminate unambiguously between *InR^{short}* and *InR^{long}*: 17405631–7, 17405651–4, and 17405784. In each case, the reference genome allele matches *InR^{short}*. Only one of the discrete polymorphisms, SNP 17405614, was variable within an allele class; the alternate and reference alleles are both associated with *InR^{short}* such that the association between the discrete allele identities and the larger haplotypes in which they are embedded (*InR^{short}* and *InR^{long}*) are approaching equilibrium ($R^2 = 0.45$). SNP 17405619 segregates at low frequency and was invariant in our Sanger sequencing data such that the *InR^{short}* and *InR^{long}* haplotypes we observed shared the same discrete allele. Both the alternate allele of SNP 17405631 and the alternate allele of 17405634–7 match both *InR^{short}* and *InR^{long}*. For polymorphism 17405637, two alternate alleles were called; the six-nt insertion matches *InR^{long}*, but the three-nt insertion is confounded with the alternate allele of 17405634–7 (which is called consistently). Thus, we ignored the three-nt insertion allele at 17405637.

Allele frequencies of SNPs at *InR* and across other sites on chromosome 3R were called from the resequencing data using CRISP (Bansal 2010) as described in Bergland et al. (2014). To test whether the *InR* locus demonstrates elevated clinality or seasonality relative to the rest of the genome, we calculated the proportion of clinal and seasonal SNPs at *InR* and compared them to random genome regions matched for length, chromosome, and inversion status. Clinality and seasonality was estimated by regressing allele frequencies on latitude or season (“spring” vs. “fall”), respectively; technical details of this analysis are available in the Supporting Information.

GENOTYPING

To genotype flies for the experimental assays, the identity of the complex *InR* indel was determined by measuring its amplified fragment length following polymerase chain reaction (PCR) with a fluorescent-tagged primer on an Applied Biosystems (Foster City, CA) 3100 capillary sequencer. PCR conditions, including primer sequences, are described in Paaby et al. (2010). That paper reported the distribution of alleles across geography and referred to the alleles by their PCR fragment lengths, which we erroneously stated as 248 bases for the high-latitude allele and 254 bases for the low-latitude allele. The actual fragment lengths using the cited primers are five bases longer. To avoid confusion, in this article we refer to these two alleles as *InR^{short}* (previously *InR²⁴⁸*) and *InR^{long}* (previously *InR²⁵⁴*). It should be noted that although fragment-length genotyping does not discriminate between the SNP alleles we characterized in or near the complex indel polymorphism (described above), only one of those SNPs (17405614) appears to be independent of the *InR^{short}* and *InR^{long}* allele classes. All evidence to date indicate that fragment-length genotyping unambiguously discriminates between consistent, but complex, haplotype classes (see Fig. 1A, B).

FLY STOCKS

To determine whether *InR^{short}* and *InR^{long}* alleles have different effects on phenotype, we generated stocks with *InR^{short}* or *InR^{long}* on the third chromosome, and for which the X and second chromosomes were replaced so that they were isogenic within and across lines. The X chromosome was derived from stock 2475 and the second chromosome was derived from stock 6326 from the Bloomington Stock Center. The genome regions surrounding *InR* on the third chromosome were randomized, from lines originally genotyped as *InR^{short}* and *InR^{long}*, across lines. To generate the stocks, we selected *InR^{short}* and *InR^{long}* parental strains without the *In(3R)Payne* inversion, crossed them, and allowed the offspring to recombine for four generations so that genetically variable regions from the parental third chromosomes were distributed as randomized blocks across lines. Fourteen isogenic stocks were subsequently established per allele by homozygosing the third chromosome via balancer extraction and genotyping the *InR* indel polymorphism. This entire scheme was performed twice, using lines derived from two independent populations: Mount Sinai, New York (40.95°N, 72.84°W) and Bowdoin, Maine (44.01°N latitude, 69.90°W longitude). Replicating across populations provided two sets of randomized genetic backgrounds in which to test the *InR* genotypes. Although four generations of laboratory-imposed recombination are unlikely to have isolated the *InR* alleles completely from the parental backgrounds, the rapid decay of linkage disequilibrium surrounding the polymorphism (Paaby et al. 2010) means that genetic variation on 3R is already randomized in natural populations.

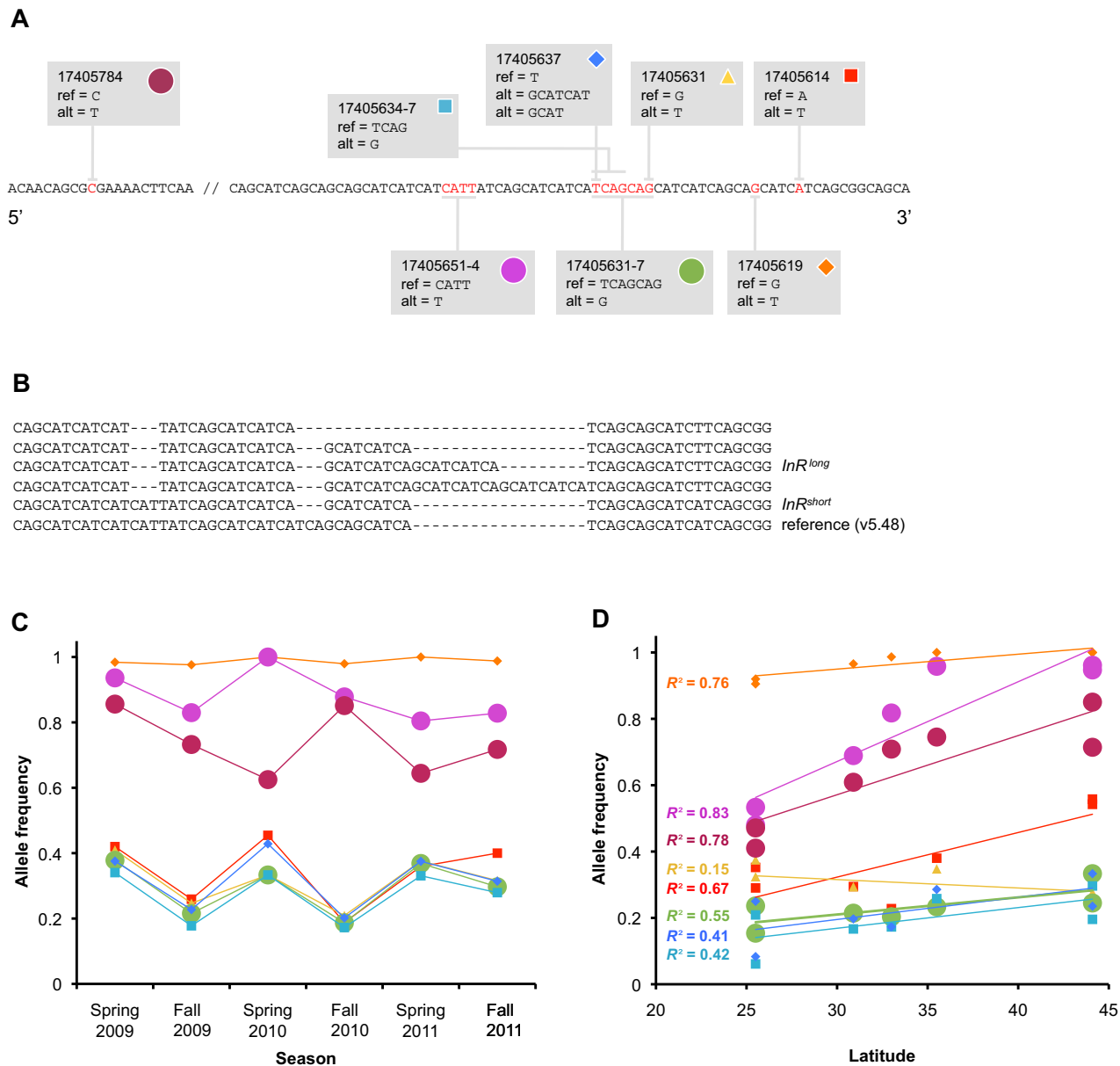


Figure 1. From pooled sequencing data, we identified eight discrete polymorphisms associated with the complex indel in the first exon of *InR* (A). Each polymorphism is labeled according to position on chromosome 3R relative to the published *D. melanogaster* reference genome (version 5.48). The discrete polymorphisms describe the complex indel haplotypes we previously characterized, which include two alleles, *InR^{short}* and *InR^{long}*, that are common at high and low latitudes, respectively (B). Polymorphisms that unambiguously discriminate between the *InR^{short}* and *InR^{long}* alleles are denoted with a large dot; in each case, the allele present in the reference genome identifies *InR^{short}*. (See Methods for relationships between the other discrete polymorphisms and *InR^{short}* and *InR^{long}*.) All eight of the discrete polymorphisms demonstrate some degree of seasonality, but four exhibit especially strong patterns in which the plotted allele increases in frequency by approximately 20% over the winter for three consecutive years (C). Alleles assigned to the *InR^{short}* allele class (large dots) generally show higher frequency in the spring and lower frequency in the fall, suggesting that overwintering may impose selection pressures consistent with high latitudes. As in our earlier report (Paaby et al. 2010), alleles assigned to the *InR^{short}* allele class also increase in frequency with latitude (D). The plotted alleles are all reference genome alleles.

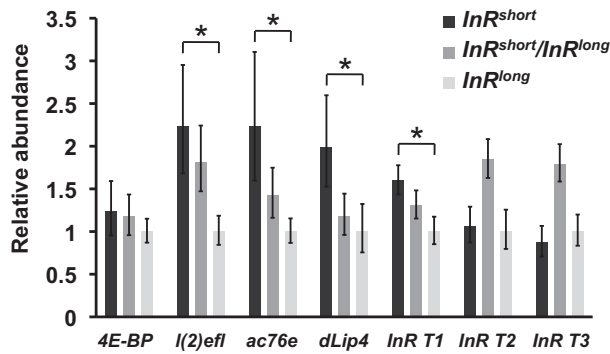


Figure 2. Relative abundance of seven transcriptional targets of dFOXO, a central transcription factor in the IIS pathway that is repressed by *InR* activity. Increased abundance of these targets indicates reduced IIS; transcript abundance for *l(2)efl*, *ac76e*, *dLip4*, and *InR T1* were significantly greater ($P < 0.05$) in *InR*^{short} samples compared to *InR*^{long}. These results are consistent with the expectation that *InR*^{short} is associated lower IIS than *InR*^{long}. Error bars show 95% confidence intervals.

InR^{short} and *InR*^{long} alleles were evaluated as homozygotes and heterozygotes, as well as with *InR* mutant and wild-type alleles derived from a laboratory strain. The *InR* mutant was the hypomorphic *InR*^{p5545} allele generated by P-element insertion in exon 1 (Tatar et al. 2001) balanced over the *TM3* chromosome (stock 11661 from the Bloomington Stock Center, *ry*⁵⁰⁶ *P{PZ}InR^{p5545}/TM3, ry^{RK} Sb¹ Ser¹*). The *TM3* balancer contains a wild-type *InR* allele (herein *InR*^{TM3}) that is intermediate in length between *InR*^{short} and *InR*^{long}: it is three nucleotides longer than *InR*^{short}, corresponding to an additional histidine, and three nucleotides shorter than *InR*^{long}, which contains an additional glutamine, as determined by Sanger sequencing.

FLIES FOR ASSAYS

Once the stocks were established, we used three methods to generate flies for the phenotype assays (derived from one or both of the independent populations). The first two tested the effects of *InR*^{short} and *InR*^{long} in a wild-type background; the third tested *InR*^{short} and *InR*^{long} effects while paired with either the *InR* hypomorphic allele or a wild-type allele in the *TM3* balancer chromosome. In the first method, we exerted nominal control over the background variation in the stocks by crossing 14 *InR*^{short} and 14 *InR*^{long} stocks in a round-robin design to generate a total of 42 lines from which we collected flies for phenotyping (14 each of *InR*^{short}/*InR*^{short}, *InR*^{long}/*InR*^{long}, and *InR*^{short}/*InR*^{long}). The crosses were conducted in standard media vials with seven virgin females (two- to five-day old) and four young males (three- to eight-day old). This design produced nonindependent replication within each genotypic class (in that chromosomes were shared across lines), and because test stocks were never mated to themselves, also ensured that no individuals were strictly

isogenic at the third chromosome. In the second method, we combined stocks (minimum of five) carrying the same allele (*InR*^{short} or *InR*^{long}) but otherwise randomized variation on the third chromosome in bottles, permitting the stocks to continue recombining freely. To rear flies for the assays, 40 virgin females (two- to five-day old) and 20 young males (three- to eight-day old) were collected and mated in fresh bottles, either within or across genotype class, to generate the two homozygous and the heterozygous genotypes. Unless otherwise specified, the phenotype assays employing this method were replicated via 10 mating bottles for each genotypic class. In the third method, we crossed 12 *InR*^{short} stocks and 12 *InR*^{long} stocks to the hypomorphic *InR*^{p5545}/*TM3* stock (in vials as in method 1). From these 24 lines, we collected progeny carrying both the *InR* hypomorphic allele and wild-type allele in the balancer chromosome for phenotyping.

PHENOTYPE ASSAYS

For all assays, flies were reared and assays were performed on standard cornmeal-molasses media at room temperature and subject to ambient light cycles. Larval density was kept low to limit overcrowding. We tested *InR*^{short} and *InR*^{long} alleles derived from Mount Sinai in all assays; we tested *InR*^{short} and *InR*^{long} alleles from Bowdoin in a subset of the assays. All *InR*^{short} and *InR*^{long} alleles tested in the presence of the *InR* hypomorph (*InR*^{p5545}) or wild-type balancer (*InR*^{TM3}) were from Mount Sinai. All statistics were performed using JMP version 7 (SAS Institute, Cary, NC). Within analyses, we used planned comparisons to test for significant differences between *InR*^{short} and *InR*^{long} genotypes. We measured 11 different traits, including life span, lifetime fecundity, early fecundity, development time, body weight, lipid content, body size, chill coma recovery, cold shock tolerance, starvation resistance, and heat shock resistance; assay details can be found in the Supporting Information.

QUANTITATIVE PCR

To test whether *InR* alleles affect IIS, we used quantitative PCR (qPCR) to determine the relative abundance of seven transcriptional targets of dFOXO, a central transcription factor within the IIS pathway (Jünger et al. 2003; Puig et al. 2003; Wang et al. 2005; Casas-Tinto et al. 2007; Flatt et al. 2008; Vihervaara and Puig 2008; Matilla et al. 2009). Total RNA was prepared from flies generated by method two from flies derived from Mount Sinai. Thirty virgin females from each of the three genotypic classes were collected over 24 h, aged on standard media, and snap frozen. RNA was extracted using RNeasy (Qiagen) and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad), following the manufacturer's protocol. Relative abundance of transcript levels was determined using an ABI 7500 Fast Real-Time PCR machine and SYBR Green PCR Master Mix (Applied Biosystems) by the DDC_T relative quantitation method. Four technical

replicates were used for each sample and relative abundance was normalized by using *GAPDH2* as an endogenous control. Other work has demonstrated that *GAPDH2* is an appropriate control for measuring relative levels of IIS (Hwangbo et al. 2004; Flatt et al. 2008). Primer sequences for the qPCR reaction are listed in the Supporting Information.

Results

CLINAL AND SEASONAL ESTIMATES OF ALLELE FREQUENCY

Previously, we showed that *InR* allele frequencies change in association with latitude on two continents, and hypothesized that this was in response to climate-driven selection (Paaby et al. 2010). Now, we ask whether the allele frequency cline in North America persists five years later and whether allele frequencies fluctuate seasonally in a single temperate population. Our previous survey identified haplotypes; here, we interrogated allele frequencies for the discrete polymorphisms that comprise those haplotypes using resequencing data. We find strong evidence of both clinality and seasonality for the indel polymorphism at *InR*, and support for a high degree of complexity in this system given that discrete sites within the haplotypes appear to respond differently to geographical and seasonal pressures (Fig. 1).

We identified all variants (SNPs or small indels) that comprise the complex indel itself and three SNPs –11, –16, and +130 nucleotides from the indel that segregate at frequencies above 5% (Fig. 1A, B). Four of these discrete polymorphisms show clear seasonality, with repeating fluctuations in allele frequency of approximately 20% between spring and fall time points over three consecutive years (Fig. 1C). The other four discrete polymorphisms are suggestive of seasonality: they demonstrate either comparable changes in amplitude but a deviation in the frequency pattern at a single time point, or a repetitive pattern at low amplitude. The discrete polymorphisms also show clines in allele frequency across latitude (Fig. 1D). We were able to unambiguously assign allele identities for three of the discrete polymorphisms to the *InR^{short}* or *InR^{long}* allele classes (Table S1), which exhibited strong allele frequency clines previously (Paaby et al. 2010). In each of these cases, changes in allele frequency across latitude replicated our earlier observations, and seasonal fluctuations in frequency for these discrete polymorphisms support the hypothesis that alleles favored at high latitude are favored during the winter.

Compared to the rest of the genome, the *InR* locus exhibits elevated patterns of allele frequency change across latitude and seasonal time. The region surrounding *InR* contains significantly more clinal polymorphisms relative to other regions of chromosome 3R matched for size and inversion status (42% vs. 20%,

respectively; $\Pr(\%InR > \%control) = 0.986$). Relative to matched control regions, the *InR* locus also contains slightly more seasonal polymorphisms (6% vs. 4.7%; $\Pr(\%InR > \%control) = 0.8$). Previous examination of linkage disequilibrium across *InR* (Paaby et al. 2010) indicates that the complex indel polymorphism is largely independent of variation elsewhere in the gene, which implies that many sites within *InR* may be targets of spatially and temporally varying selection.

ALLELE EFFECTS

We evaluated the effects of *InR* alleles on levels of IIS and multiple phenotypes and found that individuals carrying the *InR^{short}* allele, which is prevalent in high-latitude, temperate environments, exhibited lower levels of IIS, better survived cold temperature stresses and starvation, and showed sex-specific evidence for longer life span. Flies carrying the *InR^{long}* allele, which is common in low-latitude, warm environments, were associated with increased signaling and exhibited higher rates of fecundity, larger body mass, faster development time, and better survival of heat shock. These phenotypic differences are consistent with experimental reduction of IIS as well as life-history trade-offs observed in natural populations, and suggest that a single locus can act pleiotropically on a suite of fitness-related traits. We replicated some of the assays by using *InR^{short}* and *InR^{long}* alleles derived from two independent populations (Bowdoin and Mount Sinai); the results are reported separately. We also evaluated the effects of *InR^{short}* and *InR^{long}* in an *InR* hypomorphic background with reduced InR kinase activity. We found that the effects of *InR^{short}* versus *InR^{long}* are generally subtler than those of the wild-type versus hypomorph, and are consistent with our other observations of *InR^{short}* and *InR^{long}* and with prior work describing *InR* mutant alleles; a summary of these findings, including additional figures and tables (Figs. S2–S4 and Table S2), is available in the Supporting Information.

LEVELS OF INSULIN SIGNALING

To test whether the observed phenotypic differences between *InR^{short}* and *InR^{long}* are mediated by different levels of IIS, we used qPCR to measure relative abundance of seven transcriptional targets of dFOXO, a central transcription factor in the IIS pathway that is repressed by InR activity. Decreased InR activity increases target abundance (Puig et al. 2003), and transcripts for five of these (*4E-BP*, *l(2)efl*, *ac76e*, *dLip4*, and *InR T1*) were highest in *InR^{short}*, intermediate in the heterozygote, and lowest in *InR^{long}*; all but *4E-BP* showed statistical significance ($P < 0.05$) between *InR^{short}* and *InR^{long}* (Fig. 2). This pattern is robust considering that the *InR^{short}* genotype exhibited at most twofold higher levels relative to *InR^{long}*: a difference that may be biologically meaningful, and realistic for wild-type genotypes, but close to the limit of detection by qPCR methods. Two other targets, transcripts

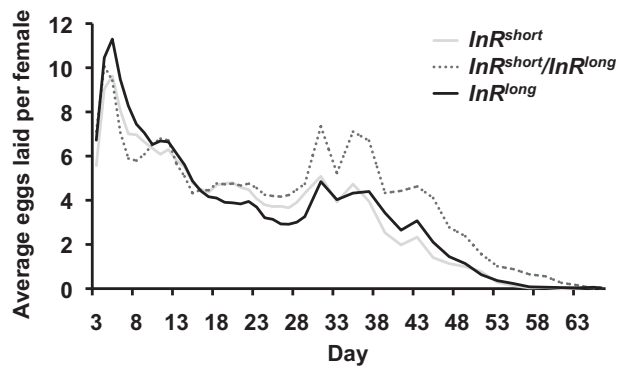
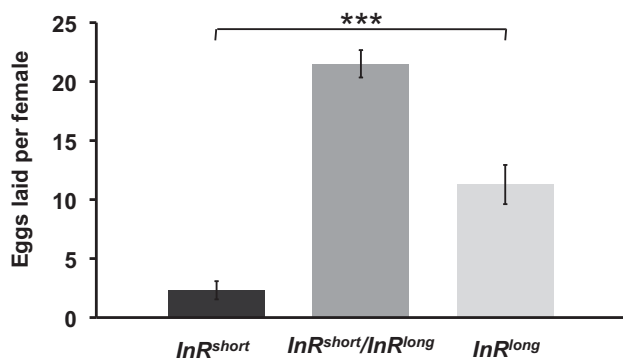
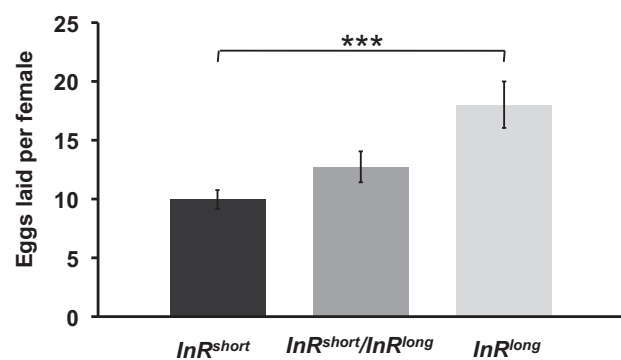
A Lifetime fecundity (Mount Sinai)**B Early fecundity (Mount Sinai)****C Early fecundity (Bowdoin)**

Figure 3. Average eggs laid per female over lifetime and in the first 12 h after mating. Lifetime fecundity was not significantly different between any genotypes except between *InR^{short}* and the heterozygote (A). However, *InR^{long}* females laid many more eggs in the first 12 h after mating than *InR^{short}* females, in comparisons from both populations (C). Error bars show 95% confidence intervals.

of *InR* itself, show no difference in abundance level between the homozygote genotypes, but significantly higher abundance in the heterozygote.

FECUNDITY

Surprisingly, the homozygous *InR* genotypes showed no significant differences in total fecundity: a fly carrying the *InR^{long}* allele laid on average only 5.3 more eggs over her lifetime than a fly carrying *InR^{short}* ($P = 0.6949$, Table 1). This was unexpected because we routinely observed substantially greater population numbers in our *InR^{long}* bottle cultures, and previous results showed that the *InR^{long}* genotype is more fecund (Paaby et al. 2010). Examination of the eggs laid per day, however, revealed that the *InR^{long}* genotype lays more eggs early in life (Fig. 3A). We performed another assay to explicitly measure early fecundity, or how many eggs young mated females laid upon first access to fresh food. Here, we found that the *InR^{long}* genotype is nearly six times more fecund than *InR^{short}* in the first 12 h of egg laying ($P < 0.0001$, Table 1 and Fig. 3B). This result was again observed in a replicate assay using flies derived from the second population ($P = 0.0005$, Table 1 and Fig. 3C).

DEVELOPMENT TIME

From egg to emerging adult, flies carrying the *InR^{long}* allele developed faster than flies carrying *InR^{short}*. This result was replicated in flies derived from two populations: in the first, males and females carrying *InR^{long}* developed an average of 6.0 h faster than those carrying *InR^{short}* ($P < 0.0001$); in the second population, development time was not significantly different among male genotypes, but *InR^{long}* females emerged an average of 18.2 h ahead of *InR^{short}* females ($P = 0.0029$, Table 1 and Fig. 4).

BODY WEIGHT AND SIZE

Because reduction in IIS reduces body size (Clancy et al. 2001; Tatar et al. 2001), we predicted that individuals carrying the *InR^{long}* allele would be larger than individuals carrying *InR^{short}*. Indeed, the average dry body weights of *InR^{long}* flies were heavier than *InR^{short}* flies. This was true for alleles tested from both populations: *InR^{long}* females and males derived from Bowdoin were 26.1% and 18.6% heavier, respectively ($P < 0.0001$ for both), whereas *InR^{long}* females and males derived from Mount Sinai were 15.5% and 10.9% heavier ($P = 0.0002$ and $P = 0.0004$, Table 1 and Figs. 5, S1). The higher mass associated with the

Table 1. Statistical results for planned comparisons and other analyses explicitly testing for significant differences between *InR^{short}* and *InR^{long}*.

	DF	SS	F	P
Mount Sinai				
Lifetime fecundity	1, 39	200.18	0.1561	0.6949
Early fecundity	1, 21	299.77	22.79	0.0001
Development time	1, 3006	17049.77	113.23	<0.0001
Dry body weight (f)	1, 45	1.10×10^{-6}	16.73	0.0002
Dry body weight (m)	1, 44	2.22×10^{-7}	14.99	0.0004
Lipid weight (f)	1, 44	0.0013	0.1361	0.7140
Lipid weight (m)	1, 43	0.0303	1.4110	0.2414
L2 wing vein (f)	1, 230	0.0157	9.0749	0.0029
L2 wing vein (m)	1, 212	0.0005	0.2759	0.5999
Chill coma (f)	1, 187	246,318.61	2.14	0.1448
Chill coma (m)	1, 207	534,075.8	16.31	<0.0001
Bowdoin				
Early fecundity	1, 27	324.82	15.55	0.0005
Development time (f)	1, 244	9383.14	9.07	0.0029
Development time (m)	1, 278	1652.35	1.42	0.2341
Dry body weight (f)	1, 37	1.42×10^{-6}	103.96	<0.0001
Dry body weight (m)	1, 39	4.03×10^{-7}	63.17	<0.0001
Lipid weight (f)	1, 36	0.0767	11.0854	0.0020
Lipid weight (m)	1, 38	0.3759	27.3649	<0.0001
L2 wing vein (f)	1, 117	0.3969	297.3210	<0.0001
L2 wing vein (m)	1, 123	0.3281	35.8582	<0.0001
Wing:thorax ratio (f)	1, 117	0.0599	35.8776	<0.0001
Wing:thorax ratio (m)	1, 123	0.0627	5.4002	0.0218
Mount Sinai	DF	χ^2	P	Risk ratio
Lifespan (f, het excluded)	1	0.0188	0.8909	0.9870
Lifespan (m, het excluded)	1	6.7336	0.0095	0.7665
Mount Sinai	Odds ratio	Reciprocal		
Cold shock (f)	0.2613	3.8275		
Cold shock (m)	0.4231	2.3636		
Starvation (f)	0.7493	1.3346		
Starvation (m)	0.5503	1.8173		
Heat shock	15.2938	0.0654		

Where sex was significant in the full model (see Tables S2 and S3), females and males were evaluated separately.

InR^{long} genotype is partially explained by lipid content. For both populations, the *InR^{long}* allele was associated with greater lipid mass than *InR^{short}* (Figs. 5, S1); however, only alleles derived from Bowdoin demonstrated a statistically significant difference when nonlipid mass was included as a covariate ($P = 0.0066$ for females, $P < 0.0001$ for males, Table 1). Wings of *InR^{long}* flies were also larger than those for *InR^{short}*, at least in the Bowdoin population. In that comparison, the average L2 wing vein length was 0.24 mm longer in *InR^{long}* females ($P < 0.0001$) and 0.12 mm longer in *InR^{long}* males ($P < 0.0001$, Table 1 and Fig. 5). In flies derived from Mount Sinai, *InR^{short}* females had the larger wings, but only by 0.02 mm ($P = 0.0029$), and *InR^{short}* and *InR^{long}* males were not different from each other ($P = 0.5999$, Table 1 and Fig. S1).

An association between large size and *InR^{long}*, the low-latitude allele, is predictable in terms of insulin signaling, but unpredictable given the observation that *Drosophila* are larger at higher latitude (Huey et al. 2000; De Jong and Bochdanovits 2003). Others have shown that proportionally larger wings relative to thorax size are associated with better flying in *Drosophila* (Hoffmann et al. 2007) and that the wing:thorax ratio increases with latitude (Karan et al. 1998), possibly because muscles in colder temperatures generate less power and proportionally larger wings can compensate for this (Gilchrist and Huey 2004). We measured thorax width to see if bigger wing:thorax ratios were associated with the high-latitude *InR^{short}* allele, but they were not. *InR^{short}* flies averaged smaller wing:thorax ratios relative to *InR^{long}* flies ($P < 0.0001$ for females, $P = 0.0218$ for males,

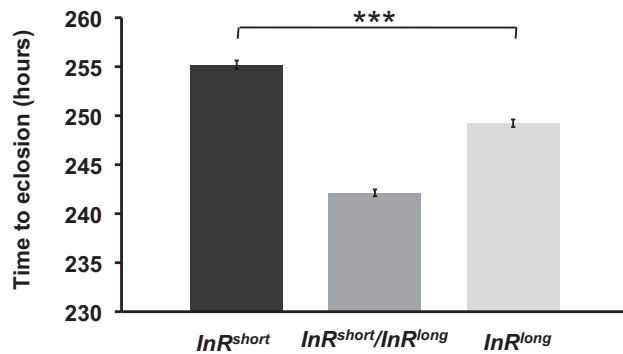
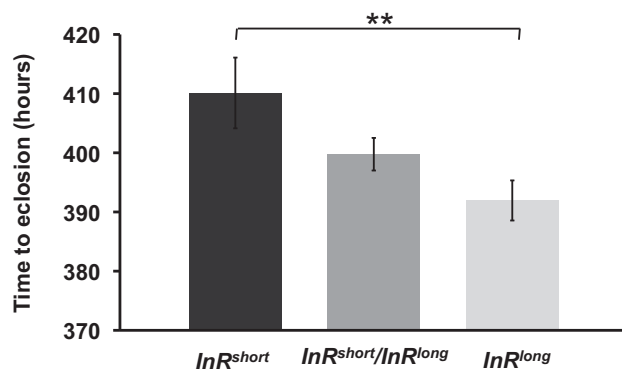
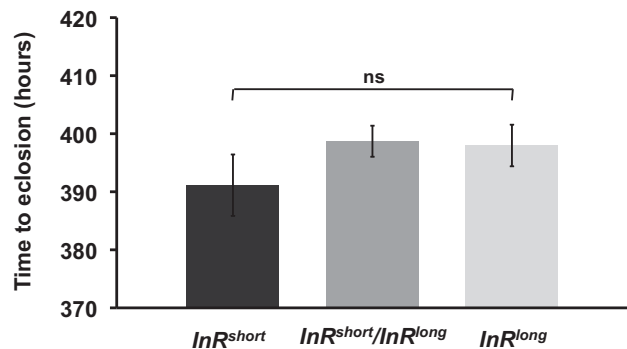
A Development time (females and males, Mount Sinai)**B Development time (females, Bowdoin)****C Development time (males, Bowdoin)**

Figure 4. Average development time, from the time the egg was laid to eclosion of the adult. Flies derived from the Mount Sinai population showed no significant differences in development time by sex, and overall *InR^{long}* flies developed faster than *InR^{short}* flies (A). However, *InR^{long}* females from Bowdoin developed faster than *InR^{short}* females (B), whereas male genotypes were not significantly different (C). Error bars show 95% confidence intervals.

Table 1 and Fig. 5). For all samples in which we measured the L2 wing vein, we also measured the L3 vein, and observed qualitatively identical results (data not shown).

STRESS TOLERANCE

Reduction in IIS confers increased resistance to stress (Giannakou and Partridge 2007), and results for three of our four stress assays accord with these findings and our hypothesis that the high-latitude *InR^{short}* allele mediates increased stress tolerance via reduced signaling (Fig. 6). Females carrying the *InR^{short}* allele recovered from chill coma on average 89 sec faster than females carrying *InR^{long}*; *InR^{short}* males recovered 123 sec faster (although this comparison was only statistically significant for males; $P = 0.1448$ and $P < 0.0001$, respectively, Table 1 and Fig. 6). Likewise, *InR^{short}* females and males exhibited 3.8 and 2.4 times the odds of surviving cold shock and 1.3 and 1.8 times the odds of surviving starvation, respectively, compared to *InR^{long}* flies. However, the *InR^{short}* allele did not correlate with higher tolerance of heat shock: instead, *InR^{short}* flies showed 15.3 times the odds of

dying in the assay compared to *InR^{long}* flies (Table 1 and Fig. 6). All four of these outcomes are consistent with presumed selection pressures in natural populations, as flies in high-latitude, temperate environments, where the *InR^{short}* allele is common, likely face cold temperatures and scarce food availability during the winter, whereas flies in low-latitude, semitropical environments, where *InR^{long}* is common, should face thermal stress from higher temperatures. However, if levels of IIS mediate these stress phenotypes, the heat shock outcome provides a unique example of correlation reversal between signaling and stress. Nevertheless, the phenotypic correlations across these traits, and the correlations between the phenotypes and latitude, are patterns consistent with other examples of reciprocal tolerance for environmentally imposed thermal stresses (Hoffmann et al. 2001, 2005).

LIFE SPAN

Insulin signaling has been established as a major regulator of longevity in many animals, including *Drosophila*: hypomorphic alleles of *InR* increase life span relative to wild-type controls

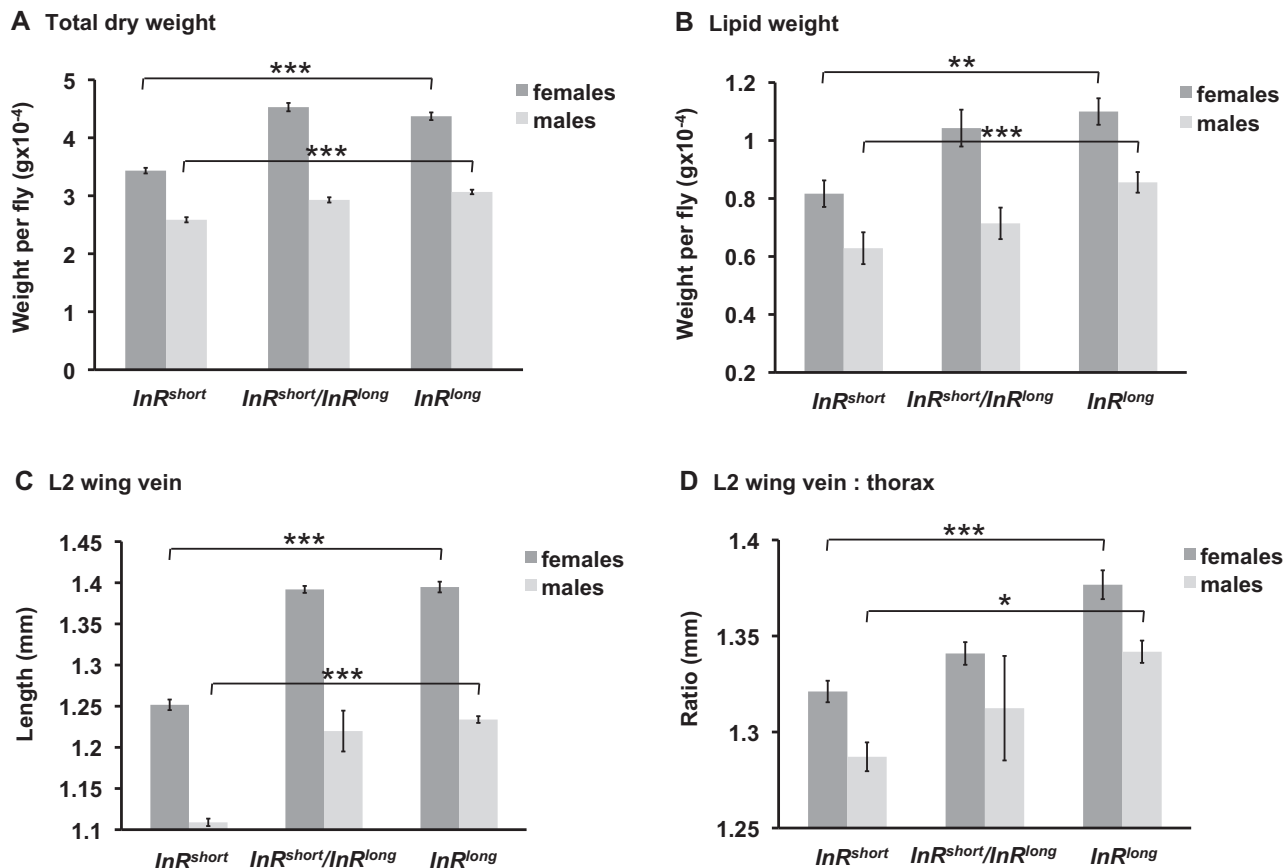


Figure 5. Average dry body weight, lipid weight, length of the L2 wing vein, and wing:thorax ratio. *InR^{short}* flies had lower dry weight (A) and lower lipid content (B) than *InR^{long}* flies. *InR^{short}* flies were also smaller, as measured by the L2 wing vein (C), and had a smaller wing:thorax ratio (D). These results are from flies derived from Bowdoin; similar findings, but of lower magnitude, were observed in flies derived from Mount Sinai (Figure S1). Error bars show 95% confidence intervals.

(Tatar et al. 2001), as do other manipulations that reduce IIS (Clancy et al. 2001; Hwangbo et al. 2004; Grönke et al. 2010). Consequently, we hypothesized that *InR^{short}* might increase life span relative to *InR^{long}*. Flies carrying the *InR^{short}* and *InR^{long}* alleles showed little difference in rates of aging over the majority of life span, although *InR^{short}* males exhibited reduced mortality late in life (Fig. 7). The lifetime survivorship of *InR^{short}* males was significantly longer than that of *InR^{long}* males, with a mortality risk ratio of 1.3 for *InR^{long}* over *InR^{short}* ($P = 0.0095$). The female mortality risk ratio for *InR^{long}* over *InR^{short}* was only 1.01, with no significant difference in longevity between these genotypes ($P = 0.8909$). Interestingly, the heterozygote of both sexes lived significantly longer ($P < 0.0001$) than either of the homozygote genotypes (Table S3). We hypothesize that the heterozygote longevity is a result of heterosis across the extended *InR*-embedded locus, a consequence of the fact that the homozygotes carried identical alleles with haplotypes extending beyond *InR*. Any general increase in fitness associated with greater heterozygosity was probably not mediated by IIS, as the heterozygotes

were associated with intermediate levels of gene expression for the majority of IIS targets.

Discussion

Our results indicate that the complex indel polymorphism in the first exon of *InR* is likely a direct target of natural selection in wild populations of *D. melanogaster*. Allele frequencies exhibit persistent latitudinal clines, change rapidly and cyclically over seasonal time scales, and the two common alleles are pleiotropically associated with multiple traits that correspond to expected pressures in distinct climatic environments. The alleles are also associated with predicted up- and downregulation of IIS, strongly suggesting that they mediate signaling behavior to effect differential trait expression. The fact that the alleles encode different amino acid sequences suggests that this occurs through changes in protein function. Overall, our findings provide an example of life-history trade-offs influenced by a complex, strongly pleiotropic polymorphism.

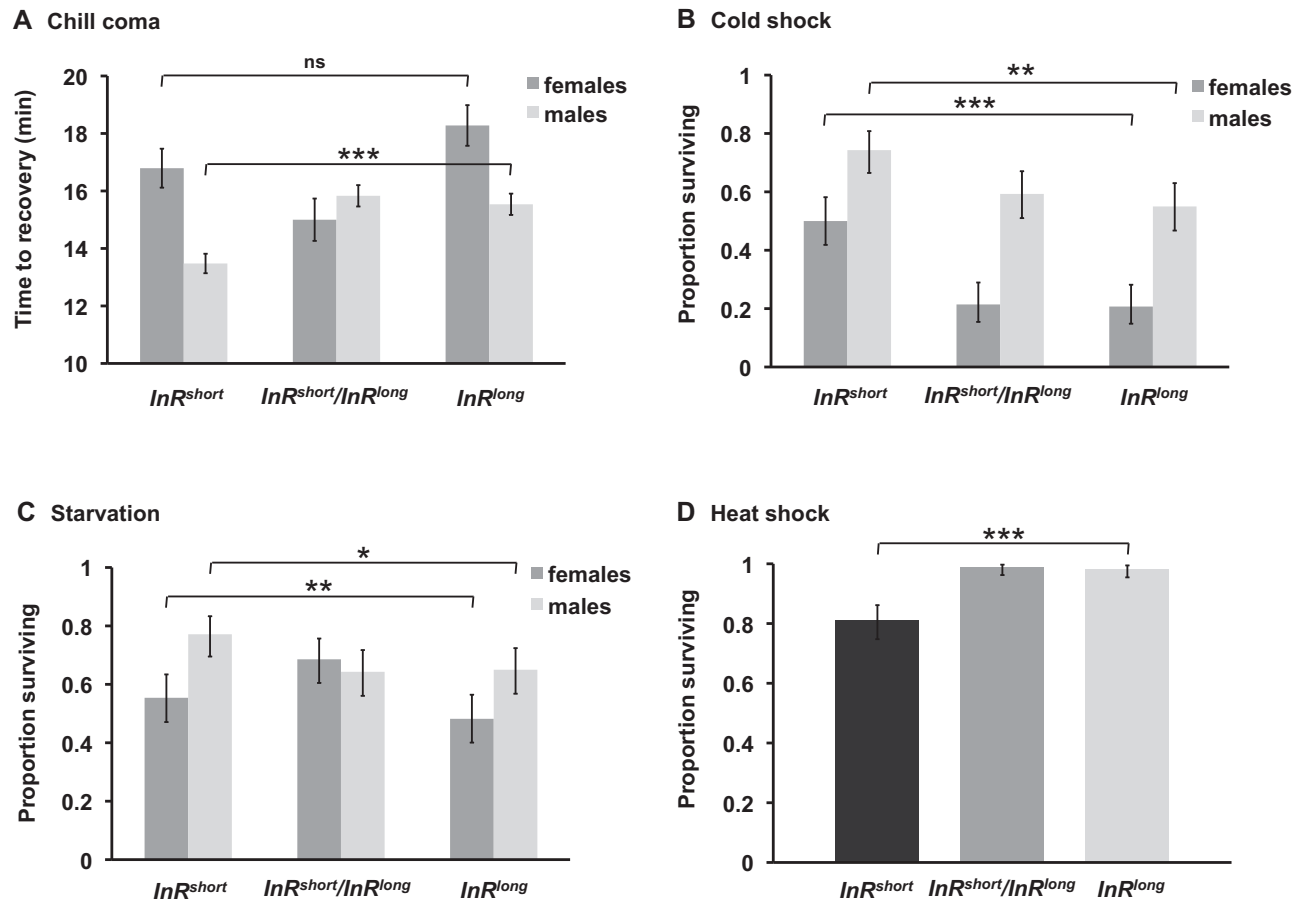


Figure 6. Average time to recovery from chill coma and proportion of flies surviving cold shock, starvation, and heat shock. Although *InR^{short}* females and males both recovered faster from chill coma than did *InR^{long}* flies, the effect was only significant for males (A). *InR^{short}* flies also better survived cold shock (B) and starvation (C), but more *InR^{long}* flies survived heat shock (D). Error bars show 95% confidence intervals.

Although the phenotypes associated with *InR^{short}* and *InR^{long}* were specific and predictive, our assays did not precisely test the effects of the glutamine and histidine changes that account for the length differences in the complex indel polymorphism. This is for two reasons. First, the *InR^{short}* and *InR^{long}* alleles themselves contain several other SNPs, each of which exhibits nonrandom patterns of allele frequency across latitude and seasonal time (Fig. 1). Only one of these (a nonsynonymous SNP at position 17405614) has the potential to confound our functional results, however, as the other SNPs did not vary between our *InR^{short}* and *InR^{long}* lines. Allele frequencies at 17405614 exhibited substantial seasonal and clinal patterns, although not as strongly as several of the discrete indel variants that comprise the complex indel polymorphism (Fig. 1). Second, genome regions flanking the complex indel polymorphism were not fully randomized due to the limits of experimentally imposed recombination. Consequently, in our test lines, the *InR^{short}* and *InR^{long}* alleles were embedded in haplotypes that extended beyond the *InR* locus. Based on prior estimates of linkage disequilibrium,

however, flanking variants should be unlinked to the focal indel polymorphism (Paaby et al. 2010). Although these other variants may be targets of selection—a conclusion supported by our observation that the entire *InR* locus exhibits elevated clinality and seasonality relative to the rest of the genome—the fact that the indel polymorphism exhibits such strong changes in allele frequency across climatic environments provides complementary evidence for allele functionality. This accords with other reports of high recombination and rapid decay of linkage disequilibrium (Mackay et al. 2012), and evidence that selection precisely targets functional loci, including *InR* (Fabian et al. 2012), in natural populations of *D. melanogaster*.

Two of the discrete polymorphisms associated with the complex indel polymorphism, 17405631 and 17405634–7, do not discriminate between *InR^{short}* and *InR^{long}* but rather discriminate between the published reference genome genotype (which is of intermediate amino acid length) and *InR^{short}* or *InR^{long}*. Both of these discrete polymorphisms show strong seasonal patterns of allele frequency, which suggests that the intermediate allele may be a

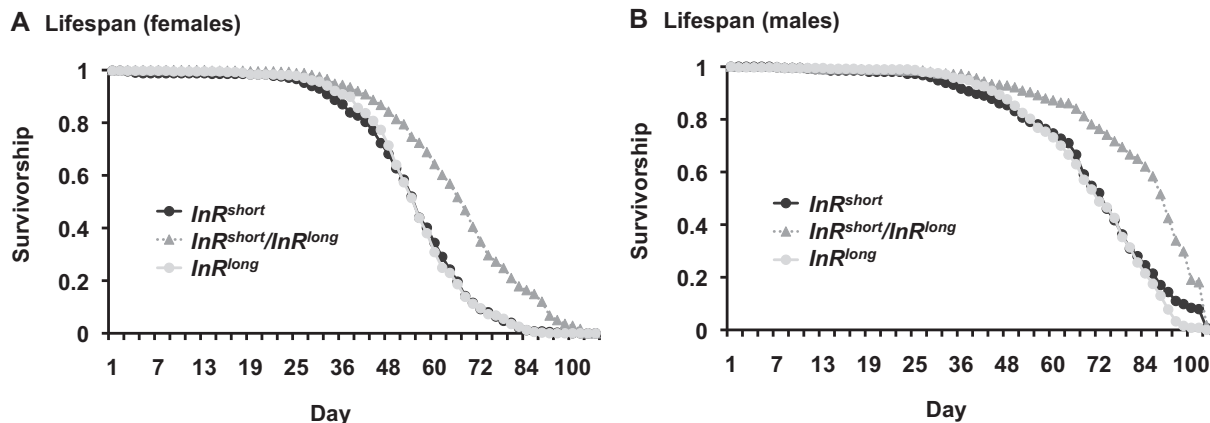


Figure 7. Survivorship curves, for females (A) and males (B). In both sexes, the heterozygote lived significantly longer than either homozygote genotype. In males, the InR^{short} genotype exhibited a reduced rate of aging late in life to produce a significant lifespan extension relative to InR^{long} , but there was no difference in longevity between InR^{short} and InR^{long} females.

target of selection as well. The discrete 17405631 and 17405634–7 alleles segregate at substantial frequencies in all populations, and in both North America and Australia the intermediate-length haplotype is the third most common allele class (Paaby et al. 2010). In our previous survey, the intermediate allele showed no cline in North America and a weak cline in Australia. Here, the discrete 17405631 and 17405634–7 alleles again show little evidence of clinality in North America (Fig. 1D and Table S1). These observations support the conclusion that seasonal and geographical environments may impose different evolutionary forces, including aspects of demography and selection, even in the face of generally concordant responses to shared climatic selection pressure (Bergland et al. 2014).

Because flies derived from high latitudes live longer than those from low latitudes (Schmidt et al. 2005a), and reduction in IIS extends life span (Clancy et al. 2001; Tatar et al. 2001; Hwangbo et al. 2004; Grönke et al. 2010), we predicted that the InR^{short} allele might be associated with longer life span than the InR^{long} allele. InR^{short} males did live significantly longer than InR^{long} males, but the magnitude of difference was small compared to other traits and there were no differences between the alleles in females. The comparatively weak longevity effect may reflect decoupling between life span and reproduction that has been observed elsewhere (Khazaeli and Curtsinger 2010)—or it may represent true functional variation. Laboratory-derived InR mutations have shown a dramatic effect on life span, but only the heterozygous combination of two hypomorphic alleles, of all genotypes tested, significantly extended life span (Tatar et al. 2001). This suggests that IIS-mediated traits may be determined by precise titration of IIS levels; it may also be the case that within the InR locus, some polymorphisms affect some traits more than others (Stern 2000). For both sexes, the heterozygote lived significantly longer than either homozygote genotype, which we interpret as a function of excess homozygosity on chromosome

3R independent of InR genotype. *Drosophila melanogaster* suffers severe inbreeding depression, which negatively affects longevity (Swindell and Bouzat 2006); as life span is a highly polygenic, quantitative trait, it stands to reason that it is the most sensitive to heterozygosity of all the traits we evaluated.

In addition to longer male life span, the InR^{short} allele was also associated with better cold and starvation tolerance and lower fecundity in females, a classic example of antagonistic pleiotropy (Williams 1957). The differences in fecundity were limited to early life, and represent variation in egg-laying rate, because total lifetime fecundity was not significantly different between the alleles. Modest differences in egg laying in early life could have massive fitness consequences in the wild, particularly on patchy substrates with larval competition, yet the competitive advantage may be undetectable in the laboratory if fecundity is measured over more than one day. Consequently, we emphasize that estimates of reproduction and other quantitative fitness traits should be examined carefully, particularly in the context of decoupling correlated traits. We also note that although the high-latitude InR^{short} allele is associated with smaller body size, in general high-latitude flies are bigger (Huey et al. 2000; De Jong and Bochdanovits 2003). If InR contributes to selection-mediated differences in body size, it either acts epistatically with other body-size loci or suffers antagonistic selection pressures across multiple fitness axes. This finding, like observations of the pleiotropic polymorphism at *neurofibromin 1* in which the high-latitude haplotype is negatively associated with wing size (Lee et al. 2013), demonstrates the complexity of selection dynamics in natural populations.

One hypothesis for strong genetic correlations among life-history traits, including the trade-off between life span and reproduction, is the presence of pleiotropic alleles. (Williams 1957; Flatt and Heyland 2011). If many genetic variants for life history are pleiotropic, then correlations among traits can never be completely dissolved. Our results implicate extensive pleiotropy, but

the complexity of the *InR* indel polymorphism limits our ability to map phenotype to specific nucleotides. The two alleles we tested, *InR^{short}* and *InR^{long}*, differ at four amino acids across a span of 16 residues (Paaby et al. 2010). Even if in isolation these sites could break pleiotropy by functioning independently, over short time scales the distinction between pleiotropy and close linkage may be inconsequential (Paaby and Rockman 2013). However, this region at *InR* clearly experiences sufficient recombination (or mutation) over longer time scales to generate a hypervariable set of alleles on which selection may act. Tests on additional *InR* alleles would help to resolve the functional roles of individual nucleotides. Moreover, our findings in support of pleiotropy may not represent the majority of life-history alleles segregating in natural populations. Other work has shown that recombination can generate genotypes with positively correlated effects for both life span and reproduction, which suggests that life-history variation in nature is likely determined by both pleiotropic and recombining nonpleiotropic loci (Khazaeli and Curtsinger 2013). Sampling genotypes from nature may represent blocks of alleles that produce the commonly observed correlations, but which can be broken under the right circumstances. Finally, although the two major alleles of the *InR* indel polymorphism showed canonical trade-offs in the laboratory, the role of these alleles in the natural environment is unknown. Whether and how they affect trait expression might vary across different environments (Fournier-Level et al. 2013), with the possibility that under some conditions, pleiotropy may present as conditional neutrality (Anderson et al. 2011b). Thus, true understanding of the role of this polymorphism in adaptive response, including fitness consequences and degree of pleiotropy, requires investigation rooted in the natural environment.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.057r9.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

- Figure S1.** Average dry body weight, lipid weight, and length of the L2 wing vein for flies derived from the Mount Sinai population.
- Figure S2.** Average early fecundity (A) and development time (B, C) for single copies of *InR^{short}* and *InR^{long}*, paired with either an *InR* hypomorphic (*InR^{p5545}*) or wild-type balancer (*InR^{TM3}*) allele.
- Figure S3.** Average dry body weight (A, B) and average lipid weight (C, D) for single copies of *InR^{short}* and *InR^{long}*, paired with either an *InR* hypomorphic (*InR^{p5545}*) or wild-type balancer (*InR^{TM3}*) allele.
- Figure S4.** Average length of the L2 wing vein (A, B) and wing:thorax ratio (C, D) for single copies of *InR^{short}* and *InR^{long}*, paired with either an *InR* hypomorphic (*InR^{p5545}*) or wild-type balancer (*InR^{TM3}*) allele.
- Table S1.** Characterization of the eight discrete polymorphisms that are associated with, or are nearby, the complex insertion–deletion polymorphism and that are segregating at appreciable frequencies in the seasonal and clinal populations.
- Table S2.** Statistical results for tests of fecundity, development, body size, and chill coma recovery for *InR* genotypes in a wild-type background.
- Table S3.** Statistical results for tests of temperature shock, starvation, and life span for *InR* genotypes in a wild-type background.
- Table S4.** Statistical results for tests of *InR^{short}* and *InR^{long}* in an *InR⁻* background.

SUPPORTING INFORMATION

Methods for comparing clinality and seasonality at *InR* to the rest of the genome

To test whether the *InR* locus (3R:17385970-17455043) demonstrates elevated clinality or seasonality relative to the rest of the genome, we calculated the proportion of clinal and seasonal SNPs at *InR* and compared them to genome-wide estimates. Clinality and seasonality was estimated by regressing allele frequencies on latitude or season (“spring” versus “fall”), respectively, using a generalized linear model with a binomial error structure; the double sampling of reads that occurs during pooled resequencing was accounted for by down-sampling reads following methods outlined in Feder et al (2012). We calculated the proportion of SNPs within the *InR* locus that were identified to be clinal (with associated clinal FDR < 0.01) or to vary repeatedly among seasons over the course of three years (with associated seasonal FDR < 0.5) and compared them to 1000 random chromosomal regions matched for length, chromosome, and inversion status for *In(3R)Payne* (which, as it suppresses recombination with standard chromosomes, affects the response to selection at individual sites). The false discovery rate for clinality and seasonality was estimated using the Benjamini-Hochberg method (1995) implemented in the *p-adjust* package in *R*. We note that the FDR value associated with seasonal SNPs here is high, and higher than the FDR cutoff used in Bergland et al. (2014). High FDR was required in order to ensure power for detecting seasonal SNPs at *InR*. Power to detect seasonality is limited, and the dataset used by Bergland et al. employed stringent filtering that removed many common SNPs due to low coverage and did not survey indel polymorphisms (including the *InR*^{short/long} alleles).

Methods for phenotype assays

Lifespan and lifetime fecundity. Bottle cages were populated with 30 virgin females and 30 males, collected over 24 hours, from lines generated by method one from the Mount Sinai population. A total of 42 bottles, comprising 14 of each genotypic class, were maintained by changing standard media egg-laying plates (without topical yeast) every day (days 1-26) or every other day (days 28 onward) until all flies were dead. Bottles were inverted, so that dead flies collected on the media plates. Dead flies and eggs laid were scored at every plate change. Lifespan was analyzed by a proportional hazards model, and cumulative fecundity was analyzed by ANOVA.

Early fecundity. Virgin females and males were collected over 6 hours, separated into single-sex vials of 5 females or 3 males each, and aged for 3 days on standard media with topical yeast. Flies were transferred in mixed-sex batches (five females and three males) to media-free vials for 4 hours, then transferred again to fresh media vials without topical yeast and permitted to lay eggs. The number of eggs laid was recorded after 12 hours and analyzed by ANOVA. Flies were generated by method two from both populations, and also with the *InR* hypomorph via method three.

Development time. Young (3-6 days old) males and females (5 flies of each sex) were transferred from standard media vials to media-free vials for four hours, then transferred again to fresh media vials and permitted to lay eggs, at densities of ≤ 30 eggs per vial, for up to four hours before being removed. Flies derived from Mount Sinai were generated via method one; flies from Bowdoin via method two, and with the hypomorph via method three. The number of adults per vial was recorded daily at 8 AM, 12 PM and 8 PM for all emerging adults, and eclosion time was analyzed by ANOVA.

Body weight, lipid content and body size. Using methods and replication schemes identical to those described for development time, eggs were reared and permitted to eclose as adults. After eclosion, adults were transferred to fresh media vials containing topical yeast and aged for 24 hours, then frozen at -80°C . To measure dry weight, frozen flies were dried for 24 hours at 55°C and weighed to 0.0001 g in single-sex batches of 7 individuals on a Sartorius microbalance (1-2 batches per original vial cross). The batches were transferred to 1.5 mL tubes and lipids were extracted by adding 400 μL diethyl ether and gently rocking the tubes for 24 hours. Flies were dried overnight at 55°C and batches re-weighed; lipid content was computed by subtraction. To estimate body size, left wings were removed from frozen flies and affixed to an index card using clear tape. Pictures of wings from a DC 300 camera (Leica) on a dissecting microscope were measured for L2 and L3 vein length using landmarks 6, 12 and 13 (Gidaszewski et al. 2009) on 3-8 males and 3-8 females from each vial cross, using tpsDigv2.12 (Rohlf 2008). By the same method thorax width was measured for the Bowdoin and hypomorph crosses. Measurements were analyzed by ANOVA.

Chill coma recovery. Freshly eclosed flies were collected over 24 hours from flies generated by method one from the Mount Sinai population, and sorted into standard media vials in batches of 5 males and 5 females each. After 3 days, males were sorted into fresh vials and aged another day. To induce chill coma, vials were covered in ice and placed at 4°C for three hours, then restored to room temperature. Time to recovery (transition to the upright position) was recorded using a video camera and analyzed by ANOVA.

Cold shock and starvation. Freshly eclosed flies were collected over 24 hours from flies generated by method one from the Mount Sinai population and method three with the hypomorph, sorted into standard media vials in batches of 5 males and 5 females, and aged for 3 days. The 42 vials generated by method one were replicated twice. To induce cold shock, vials were inverted and exposed to -20°C (air temperature) for 25 minutes. After 24 hours, individuals were scored as dead or alive. To measure starvation resistance, flies were transferred into media-free vials containing a cotton ball saturated with 2 mL of water. At 51 hours, males were scored as dead or alive; at 64 hours, females were scored as dead or alive. Both assays were analyzed by nominal logistic regression modeling the log odds of mortality/survivorship.

Heat shock. Freshly eclosed flies were collected over 24 hours and sorted into mixed-sex standard media vials of 5 males and 5 females each. Flies were generated by method two from the Mount Sinai population (15-20 replicate vials per genotypic class) and by method three. Flies were aged for 4 days and then transferred to fresh media vials. Vials were inverted, placed at 25°C, and warmed to 37°C; after reaching 37°C (approximately 20 minutes), flies were kept at 37°C for 25 minutes and then removed to room temperature to recover. After 24 hours, flies were scored as dead or alive and analyzed by nominal logistic regression modeling the log odds of mortality/survivorship.

Primer sequences for qPCR

Primer sequences for the qPCR reaction are: *GAPDH2* forward, GCGGTAGAATGGGGTGAGAC; *GAPDH2* reverse, TGAAGAGCGAAAACAGTAGC; *4E-BP* forward, GAAGGTTGTCATCTCGGATCC; *4E-BP* reverse, ATGAAAGCCCGCTCGTAGA; *l(2)efl* forward, AGGGACGATGTGACCGTGTC; *l(2)efl* reverse, CGAAGCAGACGCGTTTATCC; *ac76e* forward, CAGGATGAATGACGCCCTTTCCGG; *ac76e* reverse, ATGGACACAACACATGCCAGCAGC; *dLip4* forward, GATAGCAATGTGCGGTTGGA; *dLip4* reverse, TCATCCGTCTCCAAGGTGTG; *InR T1* forward, CACAAGCTGGAAAGA AAGTGC; *InR T1* reverse, CAAACACGTTTCGATAATATTTTTCT; *InR T2* forward, GCCTCGCACTTTGCTTATGT; *InR T2* reverse, AAAACAACGACAGCGACAA; *InR T3* forward, TTACGCCACTGCATTCGTTTC; *InR T3* reverse, ATGGCCTCTCTCTCCGTCTC.

Results for allele tests in an *InR*⁻ background

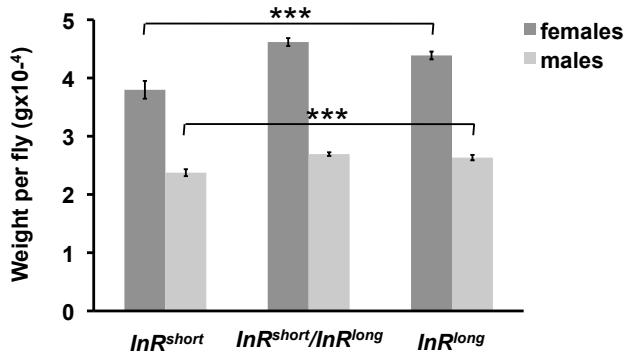
To examine how the *InR*^{short} and *InR*^{long} alleles performed in a genetic background with reduced *InR* activity, we crossed them into a stock with a hypomorphic allele (*InR*^{p5545}) over a wild-type copy of *InR* on the *TM3* balancer chromosome (*InR*^{TM3}), generating the four possible genotypic combinations. Previous work with this hypomorphic allele showed that a single copy of *InR*^{p5545} reduces *InR* kinase activity by approximately half, and not as much as a double mutant (Tatar et al. 2001). The genotypic combinations allowed us to compare single copies of *InR*^{short} to *InR*^{long}, and wild-type flies to flies carrying a single copy of the hypomorphic *InR* mutant allele.

Consistent with the expectation that *InR* function increases fecundity, flies carrying the hypomorphic allele laid fewer eggs in the first 12 hours after mating than wild-type flies ($P=0.0109$). When paired with the hypomorphic allele, *InR*^{long} females laid on average 38% more eggs than *InR*^{short} females, which is consistent with the direct allele tests but was not statistically significant in this assay, and there was no difference in egg-laying between *InR*^{short} and *InR*^{long} in the wild-type background (Table S4, Figure S2). As in the direct allele tests on Bowdoin flies, here patterns of development time were sex-specific. Females carrying the *InR* hypomorph developed 9.1 hours slower than *InR* wild-type flies ($P<0.0001$), but females carrying *InR*^{short} and *InR*^{long} were not different from each other. Males with *InR*^{short} developed 1.3

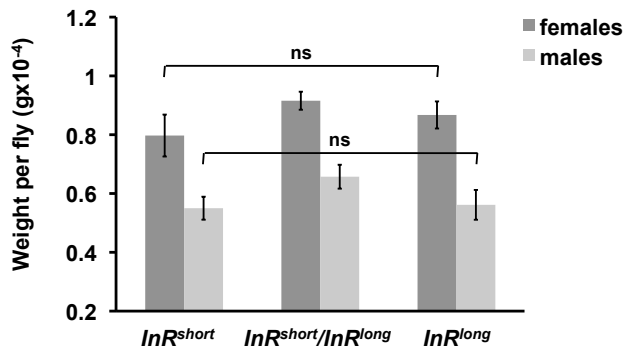
hours slower than InR^{long} males ($P=0.0064$), but males with or without the hypomorphic allele were not different from each other (Table S4, Figure S2). These patterns are consistent with the expected association of high InR activity and faster development, and the difference between the wild-type InR^{short} and InR^{long} alleles in males is much subtler than the difference between the wild-type and mutant alleles in females.

Dry weight of wild-type flies was greater than those with the InR hypomorph ($P=0.0061$ for females; $P=0.0763$ for males), consistent with the prediction that increased InR activity results in larger mass, but there were no differences between InR^{short} and InR^{long} (Table S4, Figure S3). However, single copies of InR^{short} and InR^{long} were weakly associated with lesser and greater lipid levels, as we saw in the direct allele tests ($P=0.0729$ for females; $P=0.0624$ for males), but there were no differences between InR hypomorphic and wild-type alleles (Table S4, Figure S3). The observations of InR^{short} with lower lipid level suggest that minor reduction in InR activity can reduce lipids even though the more general relationship between IIS and lipids is a negative one. This finding is in fact consistent with earlier characterization of InR mutant alleles, in which single copies of hypomorphic alleles were associated with reduced lipids (though not in our data), and double mutants with increased lipid content, relative to the wild-type (Tatar et al. 2001). Female wing sizes were not different from each other across any of the genotypic comparisons, but wings of InR^{long} males were bigger than wings of InR^{short} males ($P=0.0179$), as we saw in the direct allele tests (Table S4, Figure S4). The InR^{short} and InR^{long} alleles had no effect on the wing:thorax ratio, but both sexes had bigger ratios in the mutant background relative to the wild-type background ($P<0.0001$ for females; $P=0.0001$ for males) (Table S4, Figure S4). The correlation between the hypomorph and larger ratio suggests that decreased InR activity may increase the wing:thorax size relationship, which is what we observed in the direct allele tests as well.

A Total dry weight



B Lipid weight



C L2 wing vein

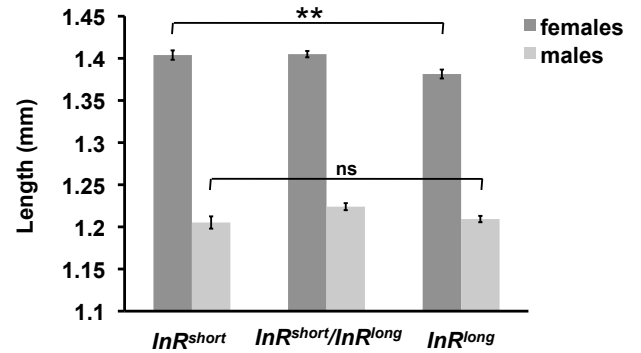
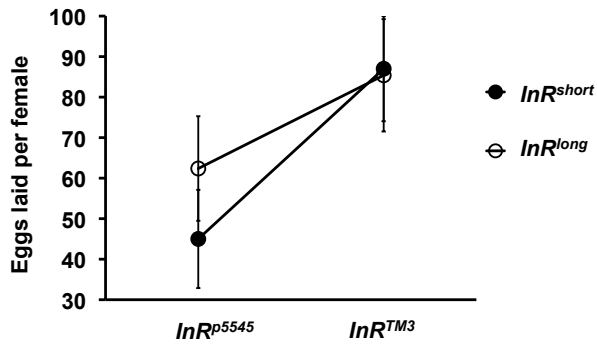
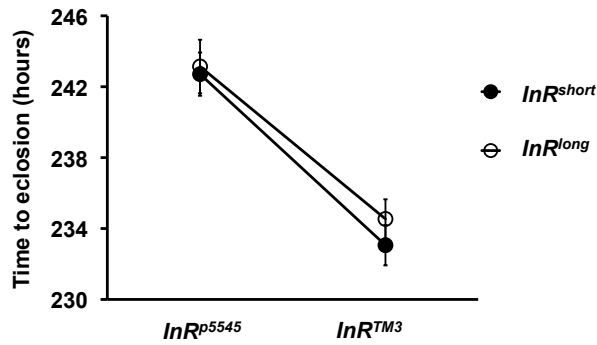


FIGURE S1. Average dry body weight, lipid weight, and length of the L2 wing vein for flies derived from the Mount Sinai population. *InR^{short}* flies had lower dry weight than *InR^{long}* flies (A), but were not significantly different in lipid weight (B). *InR^{long}* females, but not males, had significantly larger wings than *InR^{short}* (C). Error bars show 95% confidence intervals.

A Early fecundity



B Development time (females)



C Development time (males)

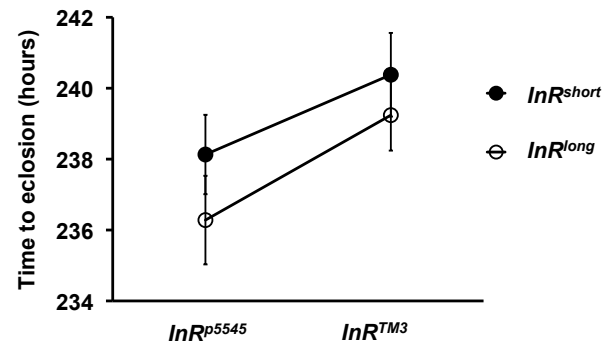
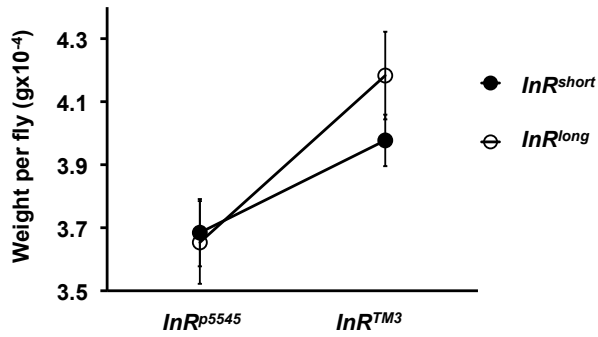
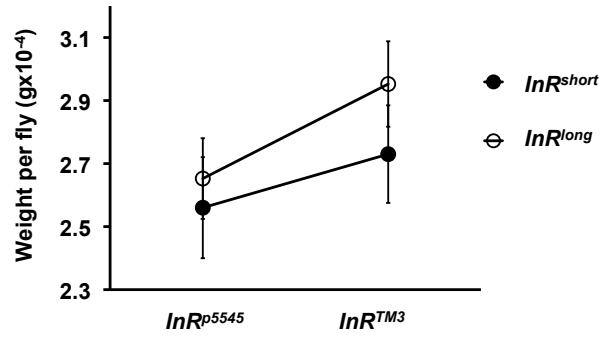


FIGURE S2. Average early fecundity (A) and development time (B, C) for single copies of *InR^{short}* and *InR^{long}*, paired with either an *InR* hypomorphic (*InR^{p5545}*) or wild-type balancer (*InR^{TM3}*) allele. Error bars show standard error of the mean.

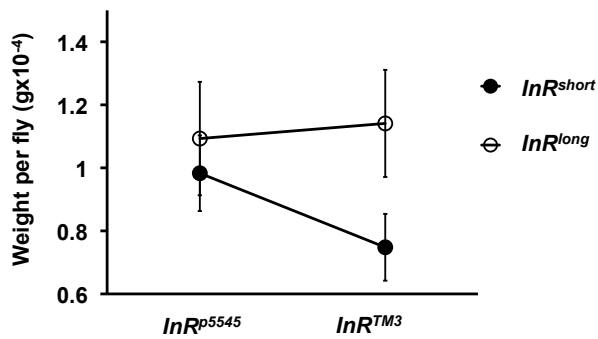
A Total dry weight (females)



B Total dry weight (males)



C Lipid weight (females)



D Lipid weight (males)

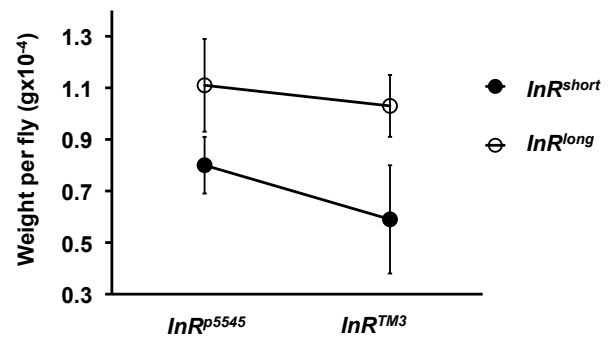
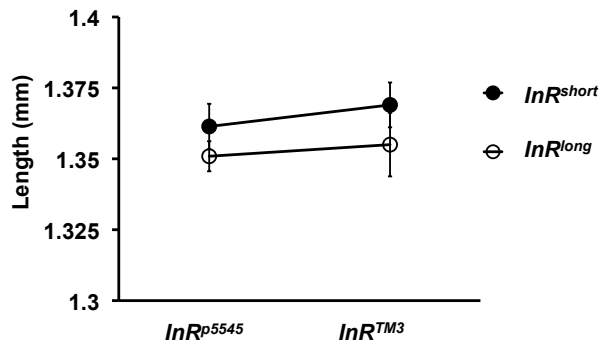
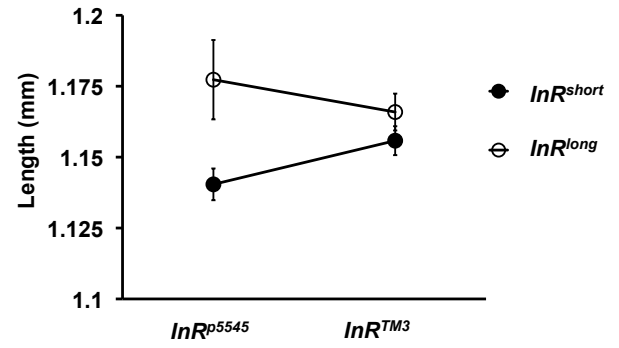


FIGURE S3. Average dry body weight (A, B) and average lipid weight (C, D) for single copies of *InR^{short}* and *InR^{long}*, paired with either an *InR* hypomorphic (*InR^{p5545}*) or wild-type balancer (*InR^{TM3}*) allele. Error bars show standard error of the mean.

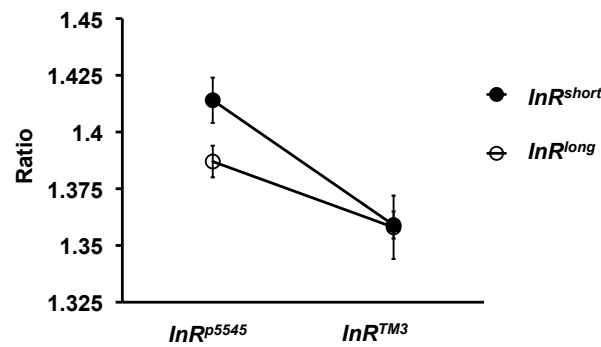
A L2 wing vein (females)



B L2 wing vein (males)



C L2 wing vein : thorax (females)



D L2 wing vein : thorax (males)

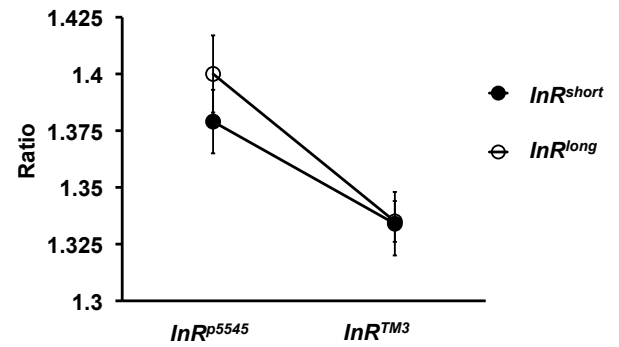


FIGURE S4. Average length of the L2 wing vein (A, B) and wing:thorax ratio (C, D) for single copies of *InR^{short}* and *InR^{long}*, paired with either an *InR* hypomorphic (*InR^{p5545}*) or wild-type balancer (*InR^{TM3}*) allele. Error bars show standard error of the mean.

TABLE S1. Characterization of the 8 discrete polymorphisms that are associated with, or are nearby, the complex insertion-deletion polymorphism and that are segregating at appreciable frequencies in the seasonal and clinal populations.

Position on 3R	Discriminates between InR^{short} and InR^{long}?	Shows seasonality?	Shows clinality?
17405614	no	suggestive	yes
17405619	no	yes (low amplitude)	yes (low magnitude)
17405631	no	yes	no
17405631-7	yes	yes	weak
17405634-7	no	yes	weak
17405637	no	yes	weak
17405651-4	yes	suggestive	yes
17405784	yes	suggestive	yes

TABLE S2. Statistical results for tests of fecundity, development, body size and chill coma recovery for *InR* genotypes in a wild-type background.

	<i>Mount Sinai population</i>					<i>Bowdoin population</i>				
	Source	DF	SS	F	P	Source	DF	SS	F	P
Lifetime fecundity	genotype	2, 39	6160.87	2.4026	0.1038					
Early fecundity	genotype	2, 21	1469.05	55.85	<0.0001	genotype	2, 27	335.23	8.02	0.0018
Development time	genotype	2, 3003	81440.13	270.48	<0.0001	genotype	2, 522	2742.59	1.24	0.2891
	sex	1, 3003	271.65	1.81	0.1793	sex	1, 522	2449.16	2.22	0.1367
	genotype x sex	2, 3003	245.64	0.82	0.4424	genotype x sex	2, 522	9928.1	4.5	0.0115
Dry body weight	genotype (f)	2, 45	2.57x10 ⁻⁶	19.51	<0.0001	genotype (f)	2, 37	2.21x10 ⁻⁶	80.95	<0.0001
	genotype (m)	2, 44	4.14x10 ⁻⁷	14	<0.0001	genotype (m)	2, 39	4.28x10 ⁻⁷	33.52	<0.0001
Lipid weight	genotype	2, 88	0.1242	3.7185	0.0282	genotype	2, 75	0.4206	19.1552	<0.0001
	sex	1, 88	0.1532	9.1744	0.0032	sex	1, 75	0.3181	28.9744	<0.0001
	genotype x sex	2, 88	0.0161	0.4833	0.6184	genotype x sex	2, 75	0.077	3.5062	0.035
	mass	1, 88	0.022	1.3187	0.2539	mass	1, 75	0.1656	15.083	0.0002
L2 wing vein	genotype (f)	2, 230	0.0253	7.3131	0.0008	genotype (f)	2, 117	0.5059	189.483	<0.0001
	genotype (m)	2, 212	0.0153	4.1532	0.017	genotype (m)	2, 123	0.3939	21.5208	<0.0001
Wing:thorax ratio						genotype (f)	2, 117	0.0627	18.7883	<0.0001
						genotype (m)	2, 123	0.0628	2.7051	0.0708
Chill coma assay	genotype (f)	2, 187	1262285	5.49	0.0048					
	genotype (m)	2, 207	829073.7	12.66	<0.0001					

TABLE S3. Statistical results for tests of temperature shock, starvation and lifespan for *InR* genotypes in a wild-type background.

	Source	DF	χ^2	P
Cold shock assay	genotype	2	45.2572	<0.0001
	sex	1	93.799	<0.0001
	genotype x sex	2	3.0473	0.2179
Starvation assay	genotype (f)	2	12.3564	0.0021
	genotype (m)	2	7.0283	0.0298
Heat shock assay	genotype	2	55.01	<0.0001
	sex	1	0.05	0.8294
	genotype x sex	2	0.65	0.7228
Lifespan	genotype (f)	2	155.2047	<0.0001
	genotype (m)	2	165.8458	<0.0001

TABLE S4. Statistical results for tests of InR^{short} and InR^{long} in an InR background. Allele refers to InR^{short} versus InR^{long} ; genotype refers to InR^{p5545} versus InR^{TM3} .

	Source	DF	SS	F	P
Early fecundity	allele	1, 21	1419.22	1.0665	0.3135
	genotype	1, 21	10378.41	7.7993	0.0109
	allele x genotype	1, 21	602.41	0.4527	0.5054
	line(allele)	24, 21	65966.5	2.0656	0.0487
Development time	allele (f)	1, 312	124.0935	0.9696	0.3255
	genotype (f)	1, 312	8017.6243	62.6441	<0.0001
	allele x genotype (f)	1, 312	11.0702	0.0865	0.7689
	line(allele) (f)	19, 312	5977.4714	2.4581	0.0008
	allele (m)	1, 336	658.434	7.5332	0.0064
	genotype (m)	1, 336	163.62	1.872	0.1722
	allele x genotype (m)	1, 336	150.351	1.7202	0.1906
	line(allele) (m)	19, 336	13405.72	8.0724	<0.0001
Dry weight	allele (f)	1, 13	0.00023442	0.3068	0.5891
	genotype (f)	1, 13	0.00816468	10.6846	0.0061
	allele x genotype (f)	1, 13	0.0000038	0.005	0.9448
	line(allele) (f)	14, 13	0.06566198	6.1377	0.0012
	allele (m)	1, 13	0.00001292	0.0036	0.9532
	genotype (m)	1, 13	0.01338558	3.7091	0.0763
	allele x genotype (m)	1, 13	0.00099214	0.2749	0.6089
	line(allele) (m)	15, 13	0.38324176	7.0798	0.0005
Lipid weight	allele (f)	1, 12	0.0915883	3.8632	0.0729
	genotype (f)	1, 12	0.0142121	0.5595	0.4538
	allele x genotype (f)	1, 12	0.0709556	2.9929	0.1092
	line(allele) (f)	14, 12	1.1486353	3.4607	0.0187
	mass (f)	1, 12	0.0049022	0.2068	0.6574
	allele (m)	1, 12	0.096919	4.2567	0.0614
	genotype (m)	1, 12	0.0000176	0.0008	0.9783
	allele x genotype (m)	1, 12	0.0019569	0.0859	0.7744
	line(allele) (m)	15, 12	1.1553349	3.3828	0.0197
	mass (m)	1, 12	0.0187888	0.8252	0.3815

L2 wing vein	allele (f)	1, 87	0.00000356	0.0022	0.963
	genotype (f)	1, 87	0.00156266	0.9506	0.3323
	allele x genotype (f)	1, 87	0.00003075	0.0187	0.8915
	line(allele) (f)	11, 87	0.03199929	1.7697	0.0717
	allele (m)	1, 84	0.00769703	5.8349	0.0179
	genotype (m)	1, 84	0.00006985	0.053	0.8186
	allele x genotype (m)	1, 84	0.00518159	3.928	0.0508
	line(allele) (m)	10, 84	0.0761658	5.7739	<0.0001
Wing:thorax ratio	allele (f)	1, 82	0.00024221	0.0896	0.7655
	genotype (f)	1, 82	0.04640658	17.1602	<0.0001
	allele x genotype (f)	1, 82	0.00459448	1.6989	0.1961
	line(allele) (f)	11, 82	0.02335652	0.7852	0.6539
	allele (m)	1, 83	0.00398441	0.9221	0.3397
	genotype (m)	1, 83	0.069767	16.1468	0.0001
	allele x genotype (m)	1, 83	0.00321297	0.7436	0.391
	line(allele) (m)	10, 83	0.09228111	2.1357	0.0303