Published in final edited form as:
Mol Ecol. 2013 May ; 22(10): 2716–2725. doi:10.1111/mec.12301.

Polymorphism in the *neurofibromin* gene, *Nf1*, is associated with antagonistic selection on wing size and development time in *Drosophila melanogaster*

Siu F. Lee[1,3], **Ying Chen Eyre-Walker**[2], **Rahul V. Rane**[1], **Caroline Reuter**[2], **Giovanna Vinti**[2], **Lea Rako**[1], **Linda Partridge**[2], and **Ary A. Hoffmann**[1]

[1]Department of Genetics and Bio21 Institute, The University of Melbourne, Australia

[2]Institute of Healthy Ageing and GEE, University College London, United Kingdom

Abstract

In many invertebrates, body size shows genetically based clines, with size increasing in colder climates. Large body size is typically associated with prolonged development times. We consider variation in the CNS-specific gene *neurofibromin 1* (*Nf1*) and its association with body size and development time. We identified two major *Nf1* haplotypes in natural populations, *Nf1-insertion-A* and *Nf1-deletion-G*. These haplotypes are characterized by a 45-base insertion/deletion (INDEL) in *Nf1* intron 2 and an A/G synonymous substitution (locus *L17277*). Linkage disequilibrium (LD) between the INDEL and adjacent sites is high but appears to be restricted within the *Nf1* gene interval. In Australia, the frequency of the *Nf1-insertion-A* haplotype increases with latitude where wing size is larger, independent of the chromosomal inversion *In(3R)Payne.* Unexpectedly, the *Nf1-insertion-A* haplotype is negatively associated with wing size. We found that the *Nf1-insertion-A* haplotype is enriched in females with shorter development time. This suggests that the *Nf1* haplotype cline may be driven by selection for development time rather than size; females from southern (higher latitude) *D. melanogaster* populations maintain a rapid development time despite being relatively larger, and the higher incidence of *Nf1-insertion-A* in southern Australia may contribute to this pattern whereas the effects of the *Nf1* haplotypes on size may be countered by other loci with antagonistic effects on size and development time. Our results point to the potential complexity involved in identifying selection on genetic variants exhibiting pleiotropic effects when studies are based on spatial patterns or association studies.

Keywords

neurofibromin; wing size; *Drosophila melanogaster*; cline; *Nf1*; candidate gene

[3]*Corresponding author*: Dr. Siu F. Lee Department of Genetics and Bio21 Institute, 30 Flemington Road, Parkville, VIC., 3010, Australia Phone: 61.3.8342348 Fax: 61.3.83442279 ronaldl@unimelb.edu.au.

Data accessibility

Raw data for clinal frequency analysis (Figure 1), wing size-*Nf1* association analyses (Figure 2) and development-*Nf1* association analysis (Figure 3) have been submitted to DRYAD: doi:10.5061/dryad.n7080.

Introduction

In many insects, adult body size (most commonly measured as wing size) shows latitudinal clines conforming to Bergmann's Rule (Arnett & Gotelli 1999; Arthur *et al.* 2008; Blanckenhorn & Demont 2004; Bryant 1977; Coyne & Beecham 1987; Huey *et al.* 2000; James *et al.* 1995). These clines are thought to be associated with climatic selection for large body size under relatively colder climates either through the juvenile stages (Blanckenhorn & Demont 2004) or directly at the adult stage (Hoffmann *et al.* 2007). Patterns of selection on size are likely to be complex, because the size of different components of an insect may be under different selection pressures (Hoffmann *et al.* 2007) and because there are pleiotropic effects of size-related genes on other traits, particularly development time (Partridge & Fowler 1993). Numerous experiments have shown that larger individuals tend to have longer development times (Prasad & Joshi 2003), and the need for rapid development is thought to drive reverse Bergmann clines where insects from cold areas emerge at a smaller size to escape a short season for breeding (Blanckenhorn & Demont 2004).

Functionally pleiotropic genes that contribute to body size variation in *Drosophila melanogaster* have been identified in genetic screens. These include the classic *Minute* mutations which occur in ribosomal protein genes, leading to suboptimal protein synthesis (Kongsuwan *et al.* 1985; Lambertsson 1998; Marygold *et al.* 2007). In addition, mutations in genes that participate in signalling pathways such as the insulin/ phosphatidylinositol 3-kinase (Ils/Pi3K), target of rapamycin (TOR), Salvador-Warts-Hippo (Hippo) pathways, result in drastic change in body size (Edgar 2006; Mirth & Riddiford 2007; Nijhout 2003; Oldham *et al.* 2000; Udan *et al.* 2003; Wu *et al.* 2003).

The search for natural polymorphisms affecting adaptive body size variation can be extended beyond genetic components of well-characterised growth pathways. Hundreds of candidate genes for climate adaptation have emerged from recent genome-wide studies of clinal variation (Chen *et al.* 2012; Fabian *et al.* 2012; Kolaczkowski *et al.* 2011; Turner *et al.* 2008). Candidate adaptive polymorphisms are found in (or near) genes of growth pathways, genes with unknown function and intergenic regions. Although powerful in identifying geographically differentiated polymorphisms, these genome-wide investigations have not been tailored for identifying polymorphisms that underpin adaptive wing size variation. So far, only two studies have investigated a specific candidate polymorphism for adaptive wing size variation; both focused on the *Dca* gene (Lee *et al.* 2011a; McKechnie *et al.* 2010).

Mutations in some genes that function in the larval central nervous system (CNS) can influence body size without obvious developmental defects (e.g., Colombani *et al.* 2005). Another candidate gene that has similar effect on size is *Neurofibromin 1* (*Nf1*), whose mammalian orthologue (~60% identity) is the causal factor of Neurofibromatosis Type 1. The function of NF1 has been studied intensively in mammalian systems (Bollag & McCormick 1991; Dasgupta & Gutmann 2003; Li *et al.* 1992; McCormick 1995). In *D. melanogaster*, the 13 kb *Nf1* gene is located at 96F9 on Chr3R approximately 1.25 Mb outside the distal break point of the chromosomal inversion *In(3R)P* at 96A18-19. *D.*

melanogaster Nf1 null mutants (generated by P-element mobilization) are 20-25% smaller and can be rescued by manipulating the cAMP-PKA pathway (The *et al.* 1997; Williams *et al.* 2001). Evidently in *D. melanogaster* NF1 is required throughout larval development to attain normal body size, whereas NF1 expression in adults is important for proper learning and memory (Guo *et al.* 2000; The *et al.* 1997). Using another set of Nf1-inactivating mutants (generated by ethyl methanesulfonate mutagenesis), Walker and co-workers showed that the size reduction phenotype can be corrected by expressing transgenic full-length *Nf1* or the NF1 GTPase-Activating Protein Related Domain (GRD) region alone in the larval CNS, and demonstrated that the action of the Ras-mediated signalling in mature neurons and Ras2-expressing cells is necessary and sufficient to restore normal body size (Walker *et al.* 2006). In addition to body size and learning disability, null mutations in *Nf1* also affect the circadian behavioural rhythmicity via the Ras/MAPK pathway (Williams *et al.* 2001). It is obvious that activities of NF1 in a narrow set of neuronal cells can have profound impact on a variety of ecologically relevant phenotypes – e.g. body size, learning/memory and circadian locomotive behaviour.

Despite being functionally correlated with size control and other ecologically relevant attributes, natural variation in *Nf1* and its association with climatic adaptation has never been explored. In this paper, we characterized the nucleotide polymorphisms at the *Nf1* locus in natural populations of *D. melanogaster*, the clinal pattern of these polymorphisms along the east coast of Australia, and their association with adaptive wing size and development time variation. Our results indicate that *Nf1* haplotypes are simultaneously associated with two traits in a sex-dependent manner and this complicates the interpretation of clinal variation and establishment of links between genetic variants and phenotypes.

Materials and methods

Isofemale line sequencing

We sequenced the *Nf1* gene region of 48 isofemale lines collected in 2008 in Innisfail, Queensland (17.31°S, 146.01°E), Maryborough, Queensland (25.32°S, 152.41°E), Crooked River Winery, New South Wales (34.44°S, 150.48°E) and Cygnet, Southern Tasmania (43.09°S, 147.07°E). Genomic DNA of 12 isofemale lines (10 females per line) from each geographic locality was extracted using the DNeasy Blood & Tissue Kit (QIAGEN: Cat. No. 69506). Overlapping PCR fragments spanning the *Nf1* gene region were amplified from each DNA sample (see Supplemental material 1, Table S1, for primer information). PCR reactions were performed in a thermal cycler using *Taq* polymerase (QIAGEN: Cat. No. 201203) following the manufacturer's protocol. The double-stranded PCR products were purified using exoSAP-IT (GE Healthcare: Cat. No. US78200). Purified PCR products were sequenced using an Applied Biosystems 3730XL 96-capillary automated DNA sequencer. Sequencing reads were assembled using CodonCode Aligner software with default settings. Sequence gaps were filled using the Celera reference sequences to generate a consensus for each line. The 48 consensus sequences were then aligned with the ClustalW program (Supplemental material 2). A custom Perl script was run to identify potential segregating sites that vary clinally (the script is available upon request).

Linkage disequilibrium analysis of Raleigh (USA) inbred line sequences

To estimate the pattern of linkage disequilibrium (LD) around the *Nf1* locus (*D.mel* r5.46 3R: 21795097-21835096), we analyzed a 40 kb genomic interval of 156 inbred *D. melanogaster* lines originated from Raleigh, North Carolina, USA (http://www.hgsc.bcm.tmc.edu/projects/dgrp/). The majority of these Raleigh lines had been sequenced using the Illumina/Solexa platform; a subset of them had also been sequenced using the Roche/454 technology. Four lines (#28, #357, #639 and #852) were excluded from the analysis due to excess differences between the Illumina/Solexa and the Roche/454 data. Sequence reads were aligned in Sequencher 4.7 (Gene Codes Corporation) and segregating sites identified. The polymorphism data were analysed using the Haps Format module in Haploview program (Barrett *et al.* 2005). We carried out LD analysis on sites that are bi-allelic with minor allele frequency more than 1%, i.e., minimum 2 occurrences in 156 sequences. The input files for Haploview analysis can be found in Supplemental materials 3 and 4.

Clinal analysis of *Nf1* allele frequencies

To determine the frequencies of the *Nf1-insertion-A* and *Nf1-deletion-G* haplotypes in the Australian *D. melanogaster* populations, a high resolution melt (HRM) based PCR assay was developed to screen a clinal DNA panel from the 2005 field collection, which comprised 16 populations along the latitudinal transect (15.47°S - 42.78°S). Clinal collection of *D. melanogaster* field samples was previously described (McKechnie *et al.* 2010). We excluded the Kingscliff population (28.25°S) from the current analysis because of low sample number (N = 9). A pair of primers was designed to flank the polymorphic site. The primer sequences were: *Nf1*_SNP_F: 5′-CCACAAGATGTCAGCTATTGTCCTG-3′ and *Nf1*_SNP_R: 5′-GTTAAAATAAATAAAACCTTACTGTG -3′. All PCR amplification and melt analysis were performed on the Roche LightCycler® 480 system. The 10 μl PCR reaction contained 1 μl of genomic DNA template (approximately 0.5% of the whole fly DNA, isolated using the standard Chelex/proteinase K method), 1 μl of each primer at 4 μM, 1 μl of the 10X reaction buffer, 0.8 μl of dNTP mix (2 mM), 0.4 μl of MgCl, 0.25 μl of the LightCycler® 2 480 High Resolution Melting Master (Roche), 0.01 μl of IMMOLASE™ DNA polymerase (Bioline), and DEPC-treated water (Invitrogen) to make up the remaining volume. Thermal cycling conditions were: 95°C for 10 minutes, 50 cycles of 95 °C for 5 seconds, 58 °C for 10 seconds, and 72 °C for 15 seconds. One fluorescence acquisition (SYBR Green channel) was obtained after each 72 °C step before the high-resolution melting (HRM) step. Products were cooled to 40 °C for 20 seconds, raised to 95 °C for 1 minute, and reduced to 65 °C. As temperature increases gradually from 65 °C to 95 °C, fluorescence data were acquired continuously. These fluorescence records were used in subsequent HRM analysis with the Genescan software (Roche). The three genotypes produced distinct melt curves after the fluorescent data were normalized. The *In(3R)Payne* variation was previously scored in the same 2005 field collection (Lee *et al.* 2011a; Lee *et al.* 2011b) using a SNP-based PCR assay (Anderson *et al.* 2005).

Genotype-phenotype association studies

Drosophila melanogaster flies were collected in Innisfail (17.31°S, 146.01°E), Queensland, Australia in 2010. We chose Innisfail because the *Nf1-insertion-A* and *Nf1-deletion-G* frequencies are similar, increasing statistical power. A mass-bred population was set up by combining F_1 progeny from 123 isofemale lines. The mass-bred population was kept at 25°C under continuous light and a moderate density. At the F_4 generation 40 replicate vials were set up, each containing 30 eggs of mixed genotype. Twelve males and 12 females from each vial were randomly chosen for wing measurement and *Nf1* genotyping. Centroid size of the adult wings was measured as described elsewhere (Rako *et al.* 2007). The second population for *Nf1*-wing size association study came from Coffs Harbour (30.27°S, 153.13°E), Australia, which has been previously described (Lee *et al.* 2011a). Wing size in the Coffs Harbour population had been used in an association study on another gene (*Dca*) in Lee *et al.* (2011a). In the current study we determined the *Nf1* genotype (A/G polymorphism at *L17277*) on the same individuals and performed similar genotype-phenotype association analysis. In other words, the phenotype data were common between Lee *et al.* (2011a) and the present study but the genotype data were distinct. Regression analysis was performed on these data with the number of the *Nf1-deletion-G* allele (0, 1, 2) as the independent variable and wing centroid size as the dependent variable. Vial effects were initially included in mixed model GLMs but these were minor and only accounted for a few percent of the variance and were ignored because they did not influence the significance of genotypic effects. T-tests (2-tailed; assuming unequal variance) were used to compare mean wing centroid size between *Nf1* genotypes (AA, GG, AG) for each sex and probabilities were corrected for multiple comparisons by the Bonferroni method.

To test for an association between *Nf1* genotype and development time, egg to eclosion time was scored in the Innisfail mass-bred population at F_{14}. We set 39 replicate vials each containing 20 eggs. At the end of pupation period vials were inspected for newly eclosed adults every 2 hours (8 am – 4 pm) for two days and one night. Collected flies at each time point were immediately sexed and preserved in 100% ethanol. The earliest eclosing (Fast Development) and latest (Slow Development) male and female, from each vial were genotyped for the A/G polymorphism at *L17277* and the 45-bp INDEL status. To score the INDEL, flanking primers were used: *Nf1*_INDEL_F: 5′-CTTCTCCCTTGTCATATCCGGTCTC-3′ and *Nf1*_INDEL_R: 5′-ACTCAAAGTAGCAACGCTCTGGCTC-3′. With such a design, the expected amplicon sizes were 152 bp for *Nf1-deletion* and 197 bp for *Nf1-insertion*. PCR amplification was performed on the Roche LightCycler® 480 system identical to the *Nf1* SNP assay. PCR products were run on 2% agarose gel (containing 0.5% Ultra High Resolution Agarose [Scientifix]) at 250 V for 25 min, stained with ethidium bromide and visualized under ultraviolet light. A contingency test (chi-square) was performed, with the null hypothesis that the proportions of the *Nf1* alleles were the same in the early and late developing groups.

Clinal expression analysis

Fifteen *D. melanogaster* mass bred populations were established using flies collected along the east coast of Australia in March/April 2008. RNA was extracted from third instar larvae from each location, converted to cDNA which was used as templates for real time PCR.

Details about site locations, sample preparation, RNA extraction, cDNA synthesis are described in (Lee *et al.* 2011a). Real time PCR (RTPCR) was performed in the LightCycler480® instrument following Takahashi *et al.* (2010). The crossing point (CP) values were extracted using the Absolute Quantification module. We used the "delta-delta CP" quantification method (Pfaffl 2001) to estimate relative gene expression, expressed as a ratio of *Nf1* (target gene) to *RpL11* (reference gene). Our calculations assumed 100% PCR efficiency for all primer combinations and in all cDNA samples. Five technical replications were performed for each gene in each cDNA sample (i.e., clinal population). Regression analysis of *Nf1* expression on latitude was performed to test for latitudinal pattern on the basal expression of *Nf1*. A T-test (2-tailed; assuming unequal variance) was performed to compare the mean relative expression of *Nf1* between the six northernmost and six southernmost populations.

Results

Nf1 haplotypes and patterns of linkage equilibrium in natural populations

Our initial sequencing survey of isofemale lines (12 lines from each of the 4 clinal populations) suggested the presence of two hypothetical *Nf1* haplotypes that vary clinally in natural populations: *Nf1-insertion-A* and *Nf1-deletion-G*. These haplotypes could be defined by a 45-base insertion/deletion (INDEL) polymorphism in *Nf1* intron 2, corresponding to *D. melanogaster* reference genome sequence r5.46 3R: 21809459-21809522, and an A/G synonymous substitutio ñ2.9 kb downstream (Supplemental material 1, Figure S1; this site is referred to as *L17277*). The exact level of linkage disequilibrium (LD) between the 45-bp INDEL and the A/G polymorphism was not determined due to uncertainty in linkage phase of the sequences in this data set. With respect to the INDEL polymorphism, comparison of the homologous genomic region among *D. melanogaster*, *D. simulans* and *D. sechellia* suggests that the insertion allele is ancestral and that the net 45 base deletion represents a deletion of 49 bases (TCCATTCTCTATTGTTAACCCTTAAACTATGTTGCCTTACTTTATGGGG) coupled with an insertion of 4 bases (GATA).

In fully sequenced North American inbred lines, pairwise linkage disequilibrium (LD) between the INDEL and 19 nearby sites is high (i.e., $R^2 > 0.5$), spanning the entire *Nf1* gene (Supplemental material 5, Figure S2). These 18 INDEL-linked single nucleotide polymorphisms (SNPs) are located in the 5′ untranslated region (UTR) (1×), coding (13×), intronic (1×), 3'UTR (1×) and intergenic (3×) regions. All 13 exonic SNPs are synonymous mutations. Except for four loci (*L14595*, *L17277*, *L19161* and *L20462*), the 45-bp insertion is associated with the preferred codon known in this species (Supplemental material 5, Table S2). In our Australian samples (Innisfail, 2010; N = 144), 100% LD was detected between the INDEL and site *L17277*, i.e. insertion is completely coupled with *L17277-A*, whereas deletion is coupled with *L17277-G*. On the basis of complete LD, haplotype status could be inferred by genotyping the A/G polymorphism at *L17277* (Supplemental material 6, Figures S3 and S4, Table S3). We therefore considered this tight linkage as *Nf1* haplotypes: *Nf1-insertion-A* and *Nf1-deletion-G*.

Clinal variation of *Nf1* haplotypes

The ancestral *Nf1-insertion-A* haplotype is present in high frequencies in Australia (average = 78%, range = 54-98%) and shows a significant positive cline (R^2 = 0.5587; t_1 = 4.21; P < 0.001) (Figure 1). We analysed the clinal pattern of *Nf1-insertion-A* within the Standard and Inverted forms of *In(3R)Payne*, a chromosomal inversion polymorphism that also displays strong latitudinal cline in Australia. For the Standard arrangement, there were 85 individuals from nine populations between 25.53°S to 42.78°S. The regression of the *Nf1-insertion-A* allele on latitude remains significant (R^2 = 0.6887; t_1 = 3.94; P = 0.006) (Figure 1). This indicates that the frequency of *Nf1-insertion-A* increases with latitude independent of *In(3R)Payne*. For the inverted arrangement, there were 112 individuals from 13 populations derived from a latitudinal range of 15.47°S to 36.67°S. The regression of the *Nf1-insertion-A* allele on latitude is not significant (R^2 = 0.0674; t_1 = 0.87; P = 0.402) (Figure 1). As the linear relationship seems to level off at the northern (low latitudes) end of the cline, the lack of *Nf1-insertion-A* cline within the Inverted arrangement could be due to the low frequency of Inverted karyotype at the higher latitudes where the *Nf1-insertion-A* cline is more pronounced (Figure 1).

Nf1 haplotypes are associated with wing size

On the basis of the observed latitudinal cline of *Nf1-insertion-A*, we anticipated that *Nf1-insertion-A* would correlate positively with large wing size. Contrary to our prediction, genotypephenotype association screens indicate that *Nf1-deletion-G* is correlated with large wing size (Figure 2). For the Innisfail (tropical; latitude ~ 17°S) population, regression analysis shows that the number of *Nf1-deletion-G* haplotypes (0, 1 or 2) is significantly associated with wing centroid size in females and males, accounting for 1.76-2.17% of the wing centroid size variance (ANOVA: females: R^2 = 0.0176; $F_{1,382}$ = 6.840; P = 0.0093; males: R^2 = 0.0217; $F_{1,382}$ = 8.472; P = 0.0038). Homozygous *Nf1-deletion-G* females (N = 69; mean centroid size ± standard error = 1.714 ± 0.0062 mm) are significantly larger than homozygous *Nf1-insertion-A* counterparts (N = 141; 1.695 ± 0.0041 mm) (t_{209} = 2.497; P <0.05; Figure 2). Likewise, homozygous *Nf1-deletion-G* males (N = 59; 1.496 ± 0.0045 mm) are significantly larger than homozygous *Nf1-insertion-A* males (N = 147; 1.476 ± 0.0032 mm) (t_{205} = 3.697; P < 0.001; Figure 2).

For the Coffs Harbour population (subtropical; latitude ~ 30°S), regression analysis shows a similar genotype-phenotype association, but more convincingly in females (ANOVA: R^2 =0.0375; F = 7.358; P = 0.0073; males: R^2 $_{1,189}$ = 0.0138; $F_{1,190}$ = 2.661; P = 0.1045). Homozygous *Nf1-deletion-G* females (N = 21; 1.714 ± 0.0090 mm) are significantly larger than homozygous *Nf1-insertion-A* females (N = 87; 1.680 ± 0.0058 mm) (t_{107} = 3.015; P < 0.01; Figure 2). In males however, we found no statistical support that homozygous *Nf1-deletion-G* has larger wing size than other genotypes. This could be due to a relatively low frequency of this genotype in the test population (N = 12). A size difference was nonetheless suggested between male *Nf1-insertion-A/Nf1-deletion-G* heterozygotes (N = 94; 1.477 ± 0.0043) and *Nf1-insertion-A* homozygotes (N = 86; 1.464 ± 0.0046) (t_{179} = 2.061; P <0.10; Figure 2).

Europe PMC Funders Author Manuscripts

Nf1-insertion-A is associated with fast development

Given that the frequency of *Nf1-insertion-A* increases with latitude and that it is negatively associated with wing size, we tested for a correlation between *Nf1-insertion-A* and development time in Innisfail mass-bred population, a well-known phenotypic antagonist of body size in *D. melanogaster*. We found that *Nf1-insertion-A* haplotype is significantly over-represented in females that develop more rapidly ($\chi^2 = 8.116$; df = 1; P = 0.0044; Figure 3). However, no such allelic enrichment was evident in males ($\chi^2 = 0.014$; df = 1; P = 0.9049; Figure 3).

Clinal expression pattern of *Nf1*

At the gene expression level, clinal pattern for *Nf1* in third instar larval stage appears non-linear but is not statistically significant in a quadratic regression ($R^2 = 0.366$; $t_2 = 2.553$; P = 0.065) (Supplemental material 7, Figure S5). No significant difference in *Nf1* expression was detected between the six northernmost (~16-21°S) and southernmost (34-43°S) populations ($t_8 = 0.6056$; P = 0.562).

Discussion

Climatic selection in *D. melanogaster* along the east coast is thought to have generated numerous latitudinal clines in traits and DNA polymorphisms (Hoffmann & Weeks 2007). Establishing a link between a DNA polymorphism and an adaptive cline in a trait generally requires (1) significant genotype-phenotype association in a large random population, and (2) consistency in geographical distribution between the candidate alleles and the adaptive phenotype along the cline, ideally coupled with (3) a functional analysis of the polymorphism showing how it might influence trait variation.

In this study, we detected a consistent *Nf1*-size association based on data from two independent populations. Association analyses of the Innisfail and Coffs Harbour populations indicate that *Nf1* haplotypes contribute a relative small proportion (1.76-2.17%) to the phenotypic variance in wing size. This level is lower than the 6% detected for the INDEL polymorphism in *Dca*, another candidate locus for adaptive wing size (Lee *et al.* 2011a). It is also known that wing size can have a relatively low heritability, estimated in an Australian *D. melanogaster* approximately 20-25% (Kennington *et al.* 2007). These findings suggest that both *Dca* and *Nf1* nevertheless could be controlling a substantial part of the genetic variance in size within a population.

However, the *Nf1*-size association results are at odds with the clinal patterns across populations. The *Nf1-insertion-A* haplotype, which reaches near fixation in temperate climates (Figure 1), is associated with smaller wing size (Figure 2). The increase in frequency of *Nf1-insertion-A* allele at high latitudes therefore runs counter to the observed wing size cline in *D. melanogaster*. This finding suggests that the *Nf1* cline must be influenced by selective pressure on traits other than wing size, whereas the observed clinal pattern of an increase in wing size at higher latitudes is due to loci unlinked to *Nf1*.

Because of the strong interaction between development time and size in *D. melanogaster*, we tested for an association between development time and the *Nf1* polymorphism.

Drosophila melanogaster populations in the Australian east coast show a weak cline in egg-to-eclosion development time, with fast development being more frequent in higher latitudes (James & Partridge 1995). Nevertheless, the absence of a development time cline (or weak cline with latitude) runs counter to expectations from wing size where larger southern (i.e., higher latitude) flies are expected to have longer development times. We therefore suspect that the clinal pattern in the *Nf1-insertion-A* allele is instead linked to the need for rapid overall pre-adult development under a shorter breeding season at higher latitudes. Hence, although *Nf1* appears to act against Bergmann's cline in Australia, its association with pre-adult development could instead be a target of selection.

Many ecological factors (e.g., temperature, seasonality, food resources, habitat etc) vary with latitude. Adaptive shifts along such a geographical gradient are likely to be caused by a combination of these factors and phenotypic tradeoffs are possible (Hoffmann *et al.* 1995). Gene pleiotropy might underlie many convoluted relationships that underlie responses to these factors. The *Nf1* haplotypes simultaneously associated with wing size and development time provide an illustration of such complexity. Based on the temperature-size rule, one would predict that the larger flies typical in the cooler latitudes would develop slower. However, the opposite is observed in nature. When flies are assayed in a common-garden environment, populations collected from cooler latitudes tend to be bigger and develop faster than those from warmer latitudes (James *et al.* 1995; James & Partridge 1995). Larger wings may provide several advantages at higher latitudes. For example, reduced wing loading may aid flight dispersal for locating food resources and mates (Hoffmann *et al.* 2007). Shorter development time on the other hand may confer other fitness benefits. These include advantages in inter-and intra-specific competition (crowding, nutrient quality, infection etc) and overcoming the short growing seasons typical in higher latitudes. James and Partridge (1995) proposed that this evolutionary conundrum could be explained by higher growth efficiency at lower temperatures (higher latitudes), but the contribution of *Nf1* haplotypes to variation in growth efficiency is unclear at this stage.

Mutant analyses have shown that sufficient *Nf1* expression during larval growth is necessary to attain normal size (Guo *et al.* 2000) but it remains difficult to define the functional roles of *Nf1* in size regulation. In a previous study, over-expressing *Nf1* in pan-neuronal tissues (driven by ELAV-GAL4) could rescue the size defect in $Nf1^{null}$ mutants (Walker *et al.* 2006). In the same mutant background, over-expressing *Nf1* in wing disc using the *engrailed* (*en*) GAL4 driver failed to restore the size phenotype (Walker *et al.* 2006). In the current study, we observed a marginal decrease (~2-5%) in wing size when *Nf1* is substantially over-expressed (~20-fold) in the developing wings using a different wing-specific driver line (A9-GAL4) (Supplemental material 8, Figure S6). This is in contrast to the much more dramatic (25%) size reduction in *Nf1* null mutants (The *et al.* 1997; Walker *et al.* 2006). Judging from the marginal local impact on size, one could also argue that wing size is rather insensitive to fluctuation in *Nf1* titre in the wing. Our results therefore indirectly support earlier findings that *Nf1* affects size primarily through the neuronal system rather than directly on the developing wings.

The link between *Nf1* haplotype and *Nf1* gene expression remains obscure. We did not find any significant difference in *Nf1* expression between tropical and temperate populations

(Supplemental material 7, Figure S5), where *Nf1* haplotype frequencies differ (Figure 1). An important limitation of the current expression analysis is that PCR efficiencies were assumed to be identical across samples and genes. A different geographical pattern in basal *Nf1* expression level might emerge if the actual primer efficiencies are empirically determined in each cDNA sample. Furthermore, there is no evidence to suggest the intronic 45-bp deletion affects splicing of intron 2, as all three genotypes produce a cDNA product of identical size (results not shown).

Given the lack of evidence at the transcriptional level, perhaps the *Nf-1-insertion-A* and the *Nf1-deletion-G* alleles differ in translation efficiency. Nine of the 13 synonymous substitutions in strong LD with the 45-bp insertion (i.e., *Nf1-insertion-A*) are the preferred codons in *D. melanogaster* (Vicario *et al.* 2007) (Supplemental material 5, Table S2). The ancestral *Nf1-insertion-A* haplotype can be considered as a cluster of preferred codons locked together by LD. It is unclear if such codon usage variation between *Nf1* haplotypes has any functional and ecological implication.

Analyses of genomic divergence between *D. melanogaster* flies from tropical and temperate regions of Australia have identified hundreds of candidate loci contributing to climatic adaptation (Kolaczkowski *et al.* 2011; Levine *et al.* 2011; Turner *et al.* 2008). Climatic adaptation is likely to involve a complex set of traits comprising many additive, synergistic, antagonistic and sex-dependent interactions. Our data in *Nf1* suggest that a genetic variant can be associated with more than one trait, and that its clinal pattern may be shaped by conflicting selective factors. These findings indicate that while the overall genomic divergence provides useful candidate adaptive polymorphisms, characterization of individual allelic variants and traits are required to expose novel gene-trait associations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Alan Rako and Jennifer Shirriffs for fly maintenance and wing size measurement, Robert Good for assistance with informatics, and Douglas Wallace for providing *UAS-dNf1* stocks. Michele Schiffer assisted with fly sampling. This work is funded by National Environmental Research Council grant NE/E006574 to LP, and Australian Research Council Discovery and Laureate Fellowship grants to AAH, and the CSIRO Science Industry Endowment Fund.

References

Anderson AR, Hoffmann AA, McKechnie SW, Umina PA, Weeks AR. The latitudinal cline in the *In(3R)Payne* inversion polymorphism has shifted in the last 20 years in Australian *Drosophila melanogaster* populations. Mol Ecol. 2005; 14:851–858. [PubMed: 15723676]

Arnett AE, Gotelli NJ. Bergmann's rule in the ant lion *Myrmeleon immaculatus* DeGeer (Neuroptera: Myrmeleontidae): geographic variation in body size and heterozygosity. Journal of Biogeography. 1999; 26:275–283.

Arthur AL, Weeks AR, Sgro CM. Investigating latitudinal clines for life history and stress resistance traits in *Drosophila simulans* from eastern Australia. J Evol Biol. 2008; 21:1470–1479. [PubMed: 18811666]

Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005; 21:263–265. [PubMed: 15297300]

Blanckenhorn WU, Demont M. Bergmann and converse Bergmann latitudinal clines in Arthropods: two ends of a continuum? Integr. Comp. Biol. 2004; 44:413–424. [PubMed: 21676727]

Bollag G, McCormick F. Differential regulation of rasGAP and neurofibromatosis gene product activities. Nature. 1991; 351:576–579. [PubMed: 1904555]

Bryant EH. Morphometric adaptation of the housefly, *Musca domestica L*., in the United States. Evolution. 1977; 31:580–596.

Chen Y, Lee SF, Blanc E, et al. Genome-wide transcription analysis of clinal genetic variation in Drosophila. PLoS One. 2012; 7:e34620. [PubMed: 22514645]

Colombani J, Bianchini L, Layalle S, et al. Antagonistic actions of ecdysone and insulins determine final size in *Drosophila*. Science. 2005; 310:667–670. [PubMed: 16179433]

Coyne JA, Beecham E. Heritability of two morphological characters within and among natural populations of *Drosophila melanogaster*. Genetics. 1987; 117:727–737. [PubMed: 3123311]

Dasgupta B, Gutmann DH. Neurofibromatosis 1: closing the GAP between mice and men. Curr Opin Genet Dev. 2003; 13:20–27. [PubMed: 12573431]

Edgar BA. How flies get their size: genetics meets physiology. Nat Rev Genet. 2006; 7:907–916. [PubMed: 17139322]

Fabian DK, Kapun M, Nolte V, et al. Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. Mol Ecol. 2012; 21:4748–4769. [PubMed: 22913798]

Guo HF, Tong J, Hannan F, Luo L, Zhong Y. A neurofibromatosis-1-regulated pathway is required for learning in *Drosophila*. Nature. 2000; 403:895–898. [PubMed: 10706287]

Hoffmann AA, Ratna E, Sgrò CM, et al. Antagonistic selection between adult thorax and wing size in field released *Drosophila melanogaster* independent of thermal conditions. J Evol Biol. 2007; 20:2219–2227. [PubMed: 17887974]

Hoffmann AA, Sgro CM, Lawler SH. Ecological population genetics: the interface between genes and the environment. Annu Rev Genet. 1995; 29:349–370. [PubMed: 8825479]

Hoffmann AA, Weeks AR. Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in *Drosophila melanogaster* from eastern Australia. Genetica. 2007; 129:133–147. [PubMed: 16955331]

Huey RB, Gilchrist GW, Carlson ML, Berrigan D, Serra L. Rapid evolution of a geographic cline in size in an introduced fly. Science. 2000; 287:308–309. [PubMed: 10634786]

James AC, Azevedo RB, Partridge L. Cellular basis and developmental timing in a size cline of *Drosophila melanogaster*. Genetics. 1995; 140:659–666. [PubMed: 7498744]

James AC, Partridge L. Thermal evolution of rate of larval development in *Drosophila melanogaster* in laboratory and field populations. J Evol Biol. 1995; 8:315–330.

Kennington WJ, Hoffmann AA, Partridge L. Mapping regions within cosmopolitan inversion *In(3R)Payne* associated with natural variation in body size in *Drosophila melanogaster*. Genetics. 2007; 177:549–556. [PubMed: 17603103]

Kolaczkowski B, Kern AD, Holloway AK, Begun DJ. Genomic differentiation between temperate and tropical Australian populations of *Drosophila melanogaster*. Genetics. 2011; 187:245–260. [PubMed: 21059887]

Kongsuwan K, Yu Q, Vincent A, et al. A *Drosophila Minute* gene encodes a ribosomal protein. Nature. 1985; 317:555–558. [PubMed: 4047173]

Lambertsson A. The *minute* genes in *Drosophila* and their molecular functions. Adv Genet. 1998; 38:69–134. [PubMed: 9677706]

Lee SF, Chen Y, Varan AK, et al. Molecular basis of adaptive shift in body size in *Drosophila melanogaster*: Functional and sequence analyses of the *Dca* gene. Mol Biol Evol. 2011a; 28:2393–2402. [PubMed: 21393605]

Lee SF, Sgro CM, Shirriffs J, et al. Polymorphism in the *couch potato* gene clines in eastern Australia but is not associated with ovarian dormancy in *Drosophila melanogaster*. Mol Ecol. 2011b; 20:2973–2984. [PubMed: 21689187]

Levine MT, Eckert ML, Begun DJ. Whole-genome expression plasticity across tropical and temperate *Drosophila melanogaster* populations from Eastern Australia. Mol Biol Evol. 2011; 28:249–256. [PubMed: 20671040]

Li Y, Bollag G, Clark R, et al. Somatic mutations in the *neurofibromatosis 1* gene in human tumors. Cell. 1992; 69:275–281. [PubMed: 1568247]

Marygold SJ, Roote J, Reuter G, et al. The ribosomal protein genes and *Minute* loci of *Drosophila melanogaster*. Genome Biol. 2007; 8:R216. [PubMed: 17927810]

McCormick F. Ras signaling and NF1. Curr Opin Genet Dev. 1995; 5:51–55. [PubMed: 7749326]

McKechnie SW, Blacket MJ, Song SV, et al. A clinally varying promoter polymorphism associated with adaptive variation in wing size in *Drosophila*. Mol Ecol. 2010; 19:775–784. [PubMed: 20074315]

Mirth CK, Riddiford LM. Size assessment and growth control: how adult size is determined in insects. Bioessays. 2007; 29:344–355. [PubMed: 17373657]

Nijhout HF. The control of body size in insects. Dev Biol. 2003; 261:1–9. [PubMed: 12941617]

Oldham S, Bohni R, Stocker H, Brogiolo W, Hafen E. Genetic control of size in *Drosophila*. Philos Trans R Soc Lond B Biol Sci. 2000; 355:945–952. [PubMed: 11128988]

Partridge L, Fowler K. Responses and correlated responses to artificial selection on thorax length in *Drosophila melanogaster*. Evolution. 1993; 47:213–226.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45. [PubMed: 11328886]

Prasad NG, Joshi A. What have two decades of laboratory life-history evolution studies on *Drosophila melanogaster* taught us? J Genet. 2003; 82:45–76. [PubMed: 14631102]

Rako L, Blacket MJ, McKechnie SW, Hoffmann AA. Candidate genes and thermal phenotypes: identifying ecologically important genetic variation for thermotolerance in the Australian *Drosophila melanogaster* cline. Mol Ecol. 2007; 16:2948–2957. [PubMed: 17614909]

Takahashi KH, Rako L, Takano-Shimizu T, Hoffmann AA, Lee SF. Effects of small *Hsp* genes on developmental stability and microenvironmental canalization. BMC Evol Biol. 2010; 10:284. [PubMed: 20846409]

The I, Hannigan GE, Cowley GS, et al. Rescue of a *Drosophila* NF1 mutant phenotype by protein kinase A. Science. 1997; 276:791–794. [PubMed: 9115203]

Turner TL, Levine MT, Eckert ML, Begun DJ. Genomic analysis of adaptive differentiation in *Drosophila melanogaster*. Genetics. 2008; 179:455–473. [PubMed: 18493064]

Udan RS, Kango-Singh M, Nolo R, Tao C, Halder G. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat Cell Biol. 2003; 5:914–920. [PubMed: 14502294]

Vicario S, Moriyama EN, Powell JR. Codon usage in twelve species of *Drosophila*. BMC Evol Biol. 2007; 7:226. [PubMed: 18005411]

Walker JA, Tchoudakova AV, McKenney PT, et al. Reduced growth of Drosophila neurofibromatosis 1 mutants reflects a non-cell-autonomous requirement for GTPase-Activating Protein activity in larval neurons. Genes Dev. 2006; 20:3311–3323. [PubMed: 17114577]

Williams JA, Su HS, Bernards A, Field J, Sehgal A. A circadian output in Drosophila mediated by neurofibromatosis-1 and Ras/MAPK. Science. 2001; 293:2251–2256. [PubMed: 11567138]

Wu S, Huang J, Dong J, Pan D. *hippo* encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with *salvador* and *warts*. Cell. 2003; 114:445–456. [PubMed: 12941273]

Europe PMC Funders Author Manuscripts

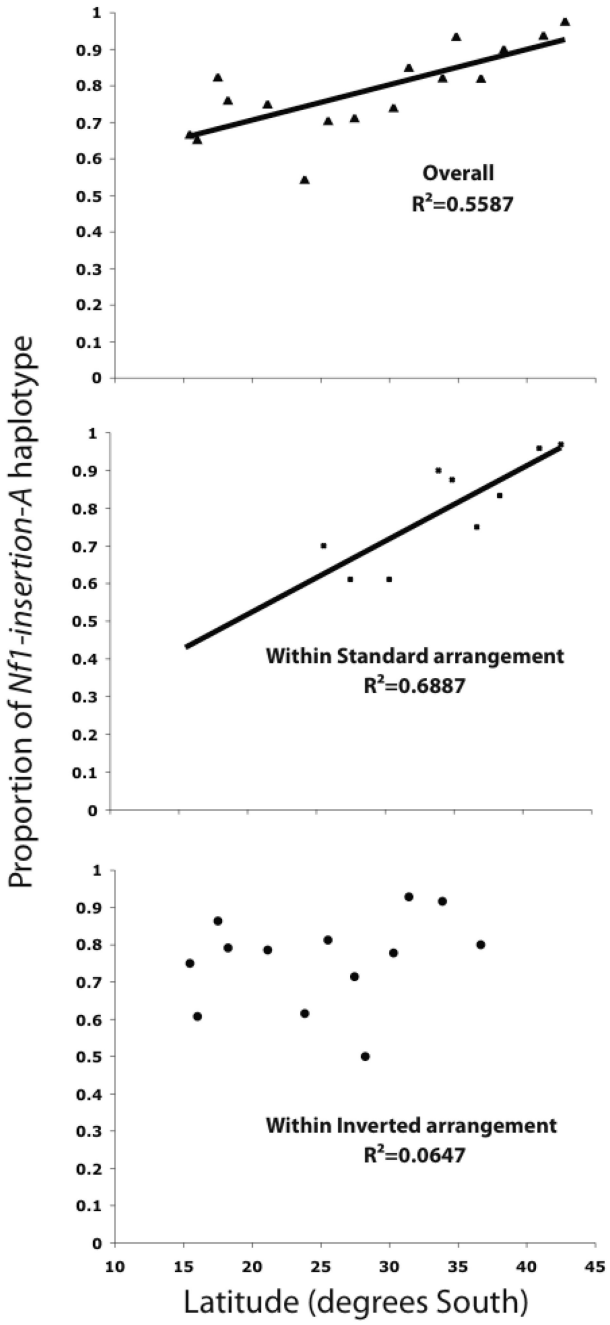

Figure 1.

Latitudinal variation in *Nf1-insertion-A* haplotype along the Australian east coast. The overall pattern is shown as well as the pattern within the inverted and standard forms of *In*(*3R*)*P*. Linear regression lines and R^2 values are also shown.

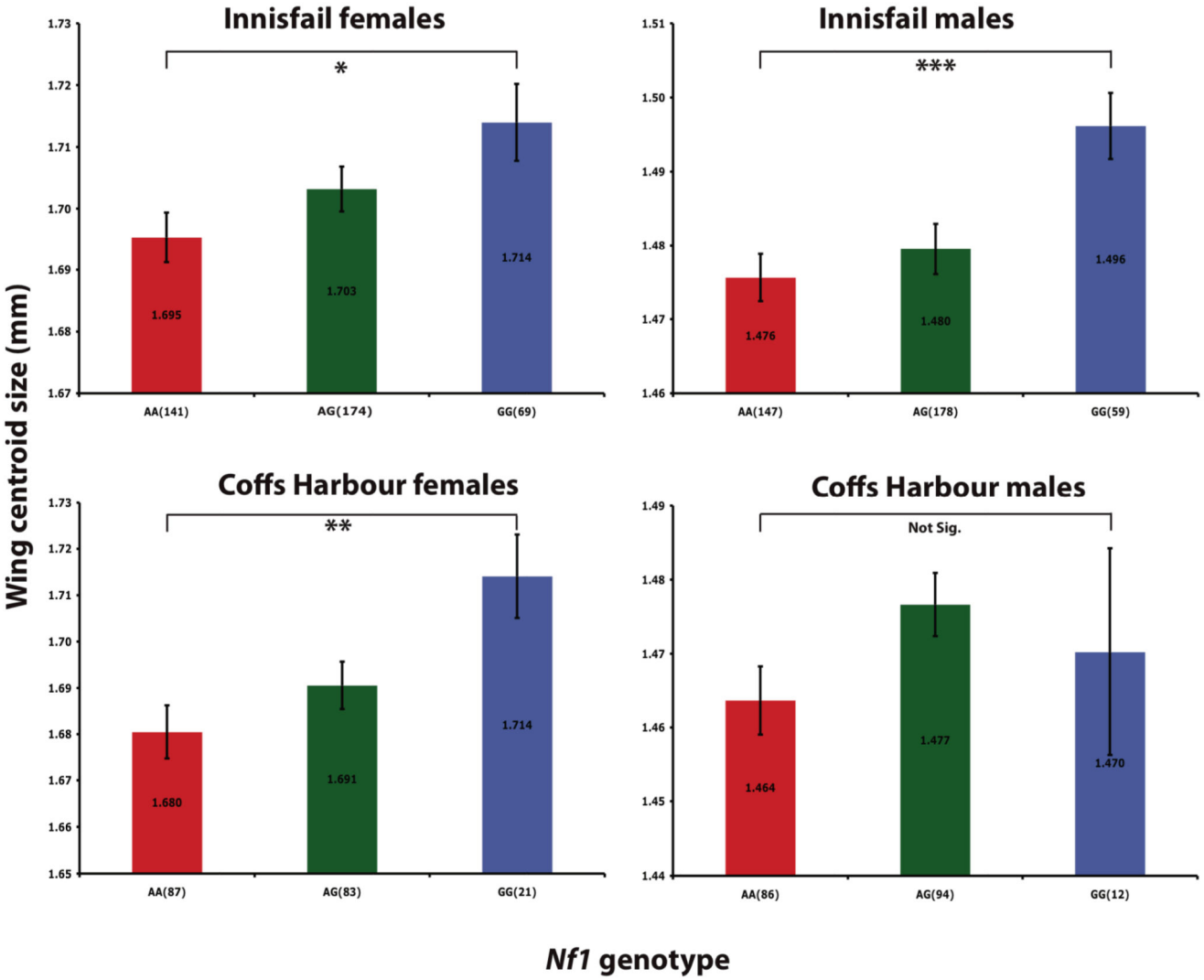

Figure 2.

Association between *Nf1* genotype and wing centroid size in Innisfail and Coffs Harbour populations. The significance of differences between the homozygous genotypes is shown (T-tests, assuming unequal variance, 2-tailed: $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***), corrected for multiple comparisons among the three genotypes). Bracketed numbers on the X-axis indicate sample sizes.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

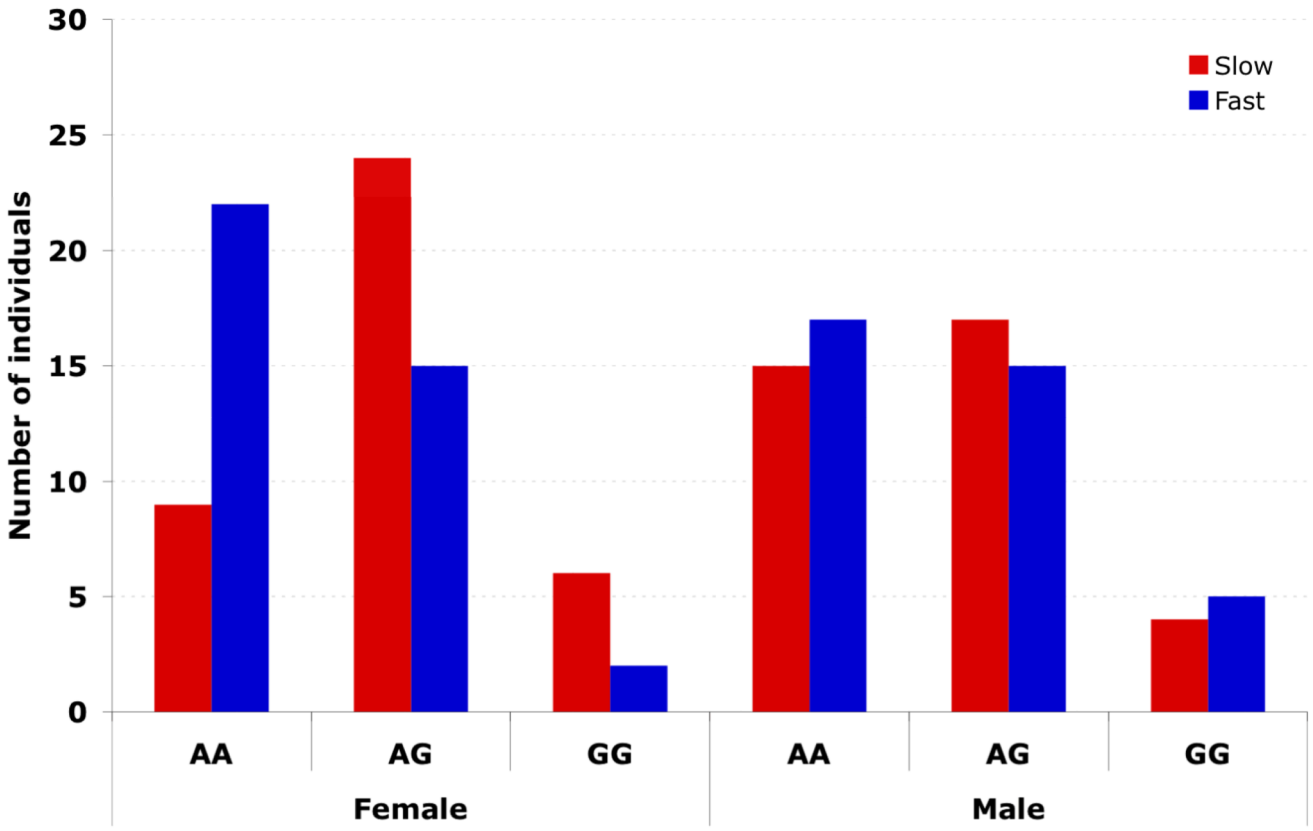

Figure 3.

Association between *Nf1* genotypes and development time. Flies from the fast and the slow developing groups were genotyped for the A/G polymorphisms at locus *L17277* and the 45-bp INDEL Sample sizes were: Male Early = 37; Male Slow = 31; Female Early = 37; Female Slow = 39. Flies used in this experiment originated from a mass-bred population from Innisfail, Queensland, Australia collected in 2010. “AA” stands for homozygous *Nf1-insertion-A*, “GG” represents homozygous *Nf1-deletion-G*, and “AG” denotes heterozygotes.