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VARIATIONS AT A QUANTITATIVE TRAIT LOCUS (QTL) AFFECT DEVELOPMENT OF BEHAVIOR IN LEAD-EXPOSED *DROSOPHILA MELANOGASTER*

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Abstract

We developed *Drosophila melanogaster* as a model to study correlated behavioral, neuronal and genetic effects of the neurotoxin lead, known to affect cognitive and behavioral development in children. We showed that, as in vertebrates, lead affects both synaptic development and complex behaviors (courtship, fecundity, locomotor activity) in *Drosophila*. By assessing differential behavioral responses to developmental lead exposure among recombinant inbred *Drosophila* lines (RI), derived from parental lines Oregon R and Russian 2b, we have now identified a genotype by environment interaction (GEI) for a behavioral trait affected by lead. Drosophila Activity Monitors (TriKinetics, Waltham, MA), which measure activity by counting the number of times a single fly in a small glass tube walks through an infrared beam aimed at the middle of the tube, were used to measure activity of flies, reared from eggs to 4 days of adult age on either control or lead-contaminated medium, from each of 75 RI lines. We observed a significant statistical association between the effect of lead on average daytime activity across lines and one marker locus, 30AB, on chromosome 2; we define this as a Quantitative Trait Locus (QTL) associated with behavioral effects of developmental lead exposure. When 30AB was from Russian 2b, lead significantly increased locomotor activity, whereas, when 30AB was from Oregon R, lead decreased it. 30AB contains about 125 genes among which are likely “candidate genes” for the observed lead-dependent behavioral changes. *Drosophila* are thus a useful, underutilized model for studying behavioral, synaptic and genetic changes following chronic exposure to lead or other neurotoxins during development.

Keywords

developmental lead exposure; developmental plasticity; behavior; quantitative trait locus; locomotor activity; *Drosophila*; developmental neurotoxicology; neurotoxin; endocrine disruptor

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Introduction

The phase-out of leaded paint and gasoline resulted in substantial reduction in mean blood lead levels in the United States, but lead exposure remains a significant public health problem (White et al. 2007). In areas where environmental lead contamination has been eliminated, individuals may still carry in their skeletons lead from prior exposure, and this lead can be mobilized during times of stress. Such times include pregnancy and lactation, during which the lead can be passed on to the fetus or infant (Gulson et al. 2003). Because lead alters mechanisms underlying developmental neuronal plasticity (Lasley et al. 2001; Toscano and Guilarte 2005; White et al. 2007; Ruden et al., in press), chronic exposure of children, even at blood lead levels below the current community action level (10 μg/dL), can result in reductions in cognitive ability (Needleman et al. 1990; Surkan et al. 2007; White et al. 2007), increased likelihood of delinquency (Canfield et al. 2003; Dietrich et al. 2001; Wright et al. 2008), development of behaviors associated with Attention Deficit Hyperactivity Disorder (Braun et al. 2006; Jones et al. 2008), changes in activity (Padich et al. 1985) and altered sensory function (Altmann et al. 1998). In addition, even at very low doses, lead is an endocrine disruptor, delaying the onset of sexual maturity in girls (Wolff et al. 2008) and in rats (Dearth et al. 2002; Dearth et al. 2004; Iavicoli et al. 2004, 2006). To complicate matters, such other environmental factors as socio-economic status and stress (Virgolini et al. 2008) can modulate the severity of the effects of exposure to lead (Bellinger, 2008a; Weiss and Bellinger 2006).

Behavioral effects of chronic lead exposure have been seen in both vertebrate and invertebrate animals; the pattern of lead-dependent behavioral effects is similar, despite variation among species in the relationship between blood levels and neuronal toxicity (Garavan et al. 2000). Chronic lead exposure impairs sensory function in chickens (Lurie et al. 2006), mice (Jones et al. 2008), rats (Fox et al. 1994,1982) and possibly monkeys (Lasky et al. 1995, 2001) as well as locomotor activity levels in monkeys (Lasky and Laughlin 2001), rats (Tang et al. 1994) and *Drosophila* (Hirsch et al. 2003). Furthermore, lead impairs learning and memory in tadpoles (Strickler-Shaw and Taylor 1991), herring gulls (Burger 1990; Burger and Gochfeld 2005), mice (Sun et al. 2005) and rats (Alber and Strupp 1996; Jett et al. 1997; Moreira et al. 2001; Morgan et al. 2000).

Long term potentiation in the hippocampus, a model system to study neuronal plasticity, is impaired by developmental lead exposure (e.g. Gilbert and Mack 1998; Gilbert et al. 1996, 1999a, b; Lasley et al. 1993). Among lead's effects in the hippocampus are changes in muscarinic modulation (Cory-Slechta and Pokora 1995; Tang et al. 2008; Wang et al. 2007), N-methyl-D-aspartate receptor function (Gilbert and Lasley 2007; Guilarte 1997; White et al. 2007), voltage-gated sodium channels (Yan et al. 2008), learning-induced activation of calcium-dependent protein kinase C (Vazquez and Pena de Ortiz 2004; Xu et al. 2005) and neurogenesis in adults (Gilbert et al. 2005; Verina et al. 2007). Other delayed effects of developmental lead exposure include a reduced ability to recover from stroke (Schneider and Decamp 2007) and an increase in the likelihood of such degenerative diseases as Alzheimer's (White et al. 2007; Wu et al. 2008; Zawia and Basha 2005) and Parkinsonism (Landrigan et al. 2005; Winkel et al. 1995).

Lead affects synaptic development and function (Cooper and Manalis 1983; Morley et al. 2003; Toscano and Guilarte 2005; White et al. 2007). Chronic lead exposure decreases the density of dendritic spines and synapses in rats (Kiraly and Jones 1982; Petit and Alfano 1979; Petit and LeBoutillier 1979) and the number of synaptic contacts formed by retinotectal projections in frogs (Cline et al. 1996) but increases dendritic spine density

(presumably synaptic sites) on cortical pyramidal cells (Patrick and Anderson 1995) and cerebellar neurons (Patrick and Anderson 2000) in cats.

Calcium plays an important role at the presynaptic terminal, where it regulates transmitter release (Zucker 1996) and synaptic plasticity (Zucker and Regehr 2002), entering through voltage-dependent channels resulting in a brief, localized, and large increase in $[Ca^{2+}]_{inside}$ which in turn triggers the release of neurotransmitter (e.g. Atlas 2001; Neher and Sakaba 2008). Lead exposure interferes with normal calcium signaling in neurons (Audesirk and Audesirk 1989; Zhang et al. 2002), affecting both the calcium-dependent enzyme neuronal nitric oxide synthase (Chetty et al. 2001; Reddy et al. 2002) and the plasma membrane calcium ATPase (PMCA) (Vazquez and Pena de Ortiz 2004). In *Drosophila* larvae, developmental lead exposure increases the Ca^{2+} transient produced by action potential trains at identified neuromuscular synapses and this intracellular Ca^{2+} signal decays more slowly than in controls. These effects are most likely due to slower calcium extrusion from lead-exposed terminals (He et al. unpublished). Calcium extrusion from synaptic terminals involves PMCA, and it appears that lead exposure inhibits this vital enzyme both in mammals (Bettaiya et al. 1996; Mas-Oliva 1989; Sandhir and Gill 1994) and in *Drosophila* (He et al. 2008 unpublished).

We recently developed *Drosophila melanogaster,* as a model system to study behavioral, neuronal and also genetic effects of chronic lead exposure during development (Hirsch et al. 2003; Morley et al. 2003). We found that at low doses, lead affects development of such behaviors as courtship, which has been shown to be experience-dependent (Hirsch and Tompkins 1994; Hirsch and Ghiradella 2004; Hirsch et al. 2001), and fecundity (Hirsch et al. 2003); behaviors, such as locomotion, are affected at somewhat higher doses (Hirsch et al. 2003). In *Drosophila* larvae lead affects development of the neuromuscular junction (Morley et al. 2003) as well as inhibiting PMCA (He et al. unpublished).

Our objective in this study was to identify a genotype by environment interaction (GEI) for a behavioral response to lead among 75 recombinant inbred (RI) lines constructed from Oregon R and Russian 2b parental strains: that is, a variation among RI genotypes in the magnitude (and/or direction) of a lead-induced behavioral change (Sambandan et al. 2008). To do this we measured locomotor activity in control and lead-exposed individuals from each line. We identified a behavioral trait (Average Daytime Activity) which shows a significant GEI and used that to define an index that quantifies the effect of lead exposure on that trait. Using this index we performed quantitative trait locus (QTL) analysis and identified a chromosomal region for which differences in the alleles from the two parental strains impacted the magnitude (and/or direction) of the index (i.e. of the lead-induced variation in behavior). A brief report of these findings has been presented (Hirsch et al. unpublished).

Materials and Methods

Subjects

Drosophila melanogaster Recombinant Inbred (RI) Lines obtained from T. MacKay (Nuzhdin et al. 1997) were used in this study. Each RI line contains a unique sample of recombinant chromosomes marked with "*roo*" transposable element insertion sites (Nuzhdin et al. 1997). Eighty-one cytological insertion sites of the *roo*-transposable element were used as molecular markers to determine the genotype of the RI lines, with an average spacing between markers of 3.2 cM on the standard map (Leips and Mackay 2000).

Experimental Design

Two control and two lead-treated males from each of 75 *roo* lines were tested simultaneously in each of six replicates over a period of twelve weeks. Approximately 80% of the flies survived to the end of the experiment. The final sample size was not significantly different for the two treatment groups (n=685 for control flies and 663 for lead treated flies; chi-square p=0.55).

Lead and Rearing Conditions

RIs were raised at 25° C on Instant *Drosophila* Medium Formula 4–24 (Carolina Biological Supply Company, Burlington, NC) mixed with either distilled water (controls) or 250 μM Lead Acetate solution (lead-treated), under 12 hour light : 12 hour dark conditions with lights-on at 10 am and lights-off at 10 pm. The behavioral experiments described below were done at the same temperature and photoperiod conditions. Exposure to leaded or control food started at egg-laying by placing approximately 25 adult flies in each vial for two days to lay eggs. Newly eclosed adults were harvested within 24 hours of eclosion and placed on fresh medium, of the same type (leaded or control) as was present during pre-adult stages, for the first four days of adult life. All animals were transferred to control medium for day five and then transferred individually, in a randomized design and under carbon dioxide anesthesia into *Drosophila* Activity Monitors (DAMs) (TriKinetics, Waltham, MA) on day six.

Behavioral Assays

DAMs count the number of times a single fly trapped in a 5 mm diameter by 65 mm-long glass tube (with food, water and air) walks through an infrared beam aimed at the middle of the tube. Food in the DAMs was a gel made from apple juice (Mott's, Rye Brook, NY) and grape agarose medium (Genessee Scientific, San Diego, CA). DAM activity data were collected from day 6 to day 12 of adult age. To allow the flies to recover from handling and anesthesia, our analysis is based data collected starting at "lights on" (10 am) of day 7. We ended the analysis at 10am on day 11, which insured that we used data from healthy flies. Data were analyzed using RhythmWatch software (Mini Mitter Company, Inc., Sunriver, OR).

Traits

DAM Activity Counts were collected and summed into 10 minute bins. We defined three traits: Average Total Activity (Day plus Night) (ATA); Average Daytime Activity (ADA), and Average Nighttime Activity (ANA).

Statistical Analysis of Treatment Effects

Lead burdens and behavioral data were subjected to analysis of variance using the General Linear Model (GLM) procedure from SPSS V14.0. The model investigated main effects for lead treatment, *roo* line, replicate and their interactions. The lead treatment by *roo* line interaction identifies genetic mediation of lead effects.

QTL Analysis

WindowsQTLCartographer, version 2.5 (Wang 2007) was used to assess the variance for each behavioral trait explained by each *roo*-marked chromosome segment to detect Quantitative Trait Loci which significantly influence the trait (the distribution of the markers on Chromosomes I, II and III are shown in Figure 1). Single Marker Analysis (SMA), Interval Mapping (IM) and Composite Interval Mapping (CIM) were used with default settings, except that 1000 permutations were run with IM and CIM to obtain p-values. The logarithmic ratio ($-2\ \ln(H_0/H_1)$) (LR) was computed for each measure, and r^2 (the

proportion of the variation explained by the QTL) was computed for IM and CIM. Only QTL that were statistically significant using all three measures (SMA, IM and CIM) were considered valid. QTL results from the CIM analysis are reported.

Lead Burden Assay

Males from the same vials of individual *roo* lines that were used for the behavioral testing described above were saved for the "lead burden" analysis. We recorded the number of surplus adults in each vial and transferred them into coded polypropylene centrifuge tubes so that the analysis could be run blind. These vials were then frozen for storage and transport. Lead burdens were obtained for 72 of the 75 strains.

Total fruit fly body lead burden was analyzed by Inductively Coupled Plasma Mass Spectrometry at the Geology Department, Union College, according to the methods described in Hirsch et al., 2003. Total number of vials tested was 522.

Results

Lead Burdens

To verify that lead exposure had been successful, we measured body lead burdens. Results of GLM analysis revealed a significant main effect ($p < 0.001$) for lead exposure, *roo* line and dose by *roo* interaction. Thus, lead treatment was effective, and there was significant genetic variability in the lead burdens of *roo* lines. The mean lead level in all the treated flies was 46.0 +/− 1.9 pg per fly, versus 0.1 +/−0.02 pg per control fly. Mean lead levels among *roo* lines treated with lead varied from 9.8 to 127.5 pg per fly.

Behavioral Effects

Treatment effects on behavior are summarized in Table 1. There were significant behavioral main effects for *roo* line and replicate for each of the three traits: Average Total Activity, Average Daytime Activity, and Average Nighttime Activity. There were no significant main effects for lead exposure for any of these traits. Differences in activity are thus most strongly affected both by genetic variations and by subtle differences between replicates (despite the standardization of the procedure). Consistent with the latter, there were significant interactions between lead treatment and replicate. In contrast, genetic variations produce stable behavioral differences; there were no significant interactions between *roo* line and replicate. One of the three traits, Average Daytime Activity (ADA), showed a significant Lead by *roo* line interaction ($p<0.05$). The third order interaction for this trait was also significant ($p<0.013$).

Even though the RI lines are derived from only two inbred parental strains, as Figure 1 shows, the control flies display considerable allelic variation in the behavioral trait (ADA) which we used in our QTL analysis.

QTL Analysis

For purposes of QTL analysis we used ADA to compute a "Lead Index" for each *roo* line from control and lead-treated strain means as a quantitative measure of the developmental effects of lead exposure: (ADA lead-treated − ADA control)/(ADA lead-treated + ADA control). Since ADA means varied significantly among *roo* lines we used a normalized measure. For purposes of the QTL analysis, we computed this ADA-based "Lead Index" for each *roo* line from control and lead-treated strain means.

Using the average Lead Index for each line, we detected one significant QTL on Chromosome 2 at 30AB (Composite Interval Mapping LR = 25.57, $r^2 = 0.26$, $p < 0.001$; Figure 2).

We used the QTL marker at 30AB to sort the RI lines into two groups, one in which the 30AB *roo*-marked chromosome segment was from the Russian 2b parental line (N = 502), and the other in which it was from Oregon R (N = 790). Figure 3 shows the distribution of the mean Lead Index for RI lines from each of the two groups, with values ranked from largest to smallest. Note that in each group there is a range from positive to negative, but the distribution of Lead Index scores differs between groups.

To verify that genetic variation linked to the roo line marker at 30AB influenced the magnitude (and/or direction) of lead-induced changes in ADA, we performed a GLM analysis for ADA using the same two groups. There was no significant main effect for lead exposure ($F = 0.609$; $df = 1$; $p > 0.05$), or parental genotype ($F = 0.424$; $df = 1$; $p > 0.05$). However, there was a significant lead exposure by parental genotype interaction ($F = 8.173$; $df = 1$; $p < 0.005$). As Figure 4 shows, when the parental line marker allele is from Russian 2b, lead exposure significantly increases ADA ($F = 6.19$; $df = 1$; $p<0.015$); in contrast, although ADA decreases with lead exposure when the parental line marker at 30AB is from Oregon R, there is no significant effect of lead exposure ($F = 2.16$; $df = 1$; $p > 0.05$). Thus, genetic variation at the QTL identified with the lead index affected the lead-induced changes in a complex behavioral trait: ADA.

To determine whether the variation in lead burden among *roo* lines could account for the variation in ADA or in Lead Index, we computed the correlation between RI strain means for lead burden and ADA, as well as for lead burden (for lead-treated flies) and Lead Index. The correlations were not significant ($r = 0.024$, $p = 0.77$, and $r = 0.111$, $p = 0.36$ respectively). Thus strain variation in lead burden could not account for strain variation in either ADA or Lead Index.

Discussion and Conclusions

Significant RI line differences in response to developmental lead exposure were found for average daytime activity. Approximately 25% of the lead-induced change in this behavior was explained by a significant QTL at 30AB on Chromosome 2. This effect is independent of strain differences in lead burden.

To the best of our knowledge, this is the first identification of a portion of the *Drosophila* genome that is involved in a behavioral response to lead. We demonstrated a significant Gene by Environment Interaction for a complex behavior (Lead Index) and by using the *roo* lines we were able to locate a genetic site on Chromosome 2, 30AB, involved in that interaction. We are thus able, in this species, to study behavioral (Hirsch et al. 2003), synaptic (He et al. unpublished; Morley et al. 2003) and now genetic changes resulting from chronic lead exposure during development.

Whole-genome microarrays are being used to measure changes in gene expression in response to various doses of a toxin or drug (reviewed in Foster et al. 2007 and Ruden 2007). However, since hundreds or even thousands of genes may undergo toxin-dependent changes in expression, it is difficult to take the next step and validate the results through follow-up genetic or molecular studies. RI analysis can provide an *a priori* method for identifying chromosomal regions where gene expression changes are most relevant to specific traits of interest. This study has identified a region of the *Drosophila* genome linked to the observed lead-dependent change in locomotor activity. There are approximately 125 genes closely linked to the *roo* marker at 30AB (Wilson et al. 2008). Some of the

mechanisms these genes mediate include: nucleic acid binding (*numb*), transcription regulation (*tai*), signal transduction (*Toll-4*), defense responses (*Toll-4* and *CG3759*), neurotransmitter secretion (*Gdi*), development of nervous system (*numb*), voltage-gated potassium channel activity (*CG34366*), ferroxidase activity (*CG3759*), iron ion transport (*CG3759*), and copper ion binding (*CG3759*).

Our results extend the growing list of traits shown to display significant allelic variation at identified QTL in these same *roo* lines. These include sternopleural bristle number (Gurganus et al. 1998,1999), reproductive performance (Fry, Nuzhdin et al. 1998), ovariole number, body size, early fecundity, competitive fitness and life span (Vieira et al. 2000; Wayne et al. 2001), olfactory behavior (Fanara et al. 2002), starvation resistance (Harbison et al. 2005), mating behavior (Moehring and Mackay 2004), longevity (Mackay 2002) and locomotor activity (Jordan et al. 2007). These studies demonstrate that the *roo* lines display significant allelic variation for many complex traits and represent a useful model for further analysis. They also suggest that much more genetic variation exists beyond these identified QTL since the *roo* lines capture allelic differences derived from only two inbred parent strains, neither of which was selected for particular behavioral responses other than reduced mating behavior in the Russian 2b parent strain (Kaidanov et al. 1991). Analysis of the same trait in independently derived *Drosophila* lines should reveal additional genetic variation for these traits, and might also provide independent confirmation for any QTL shared in common with the *roo* lines. Morgan and Mackay (2006) used the *roo* lines to identify seven QTL affecting thermal tolerance, and compared them to four QTL identified by Norry et al. (2004) in populations of different geographic origin that were artificially selected for response to thermal stress. Two QTL co-localized between the two studies, and the rest did not, suggesting that QTL analysis of complex quantitative traits in *Drosophila* in a single population can produce significant results that may generalize to other populations, but also may represent just a fraction of potential genetic variation for the trait. In the present study, our QTL associated with altered daytime activity in response to lead exposure did not match any of the four QTL identified by Jordan et al. (2006) for locomotor reactivity.

Using a different set of 41 inbred lines derived from a wild population, Sambandan et al. (2008) were able to identify and confirm, with quantitative complementation tests, genes involved in olfactory behavior of flies reared in different food sources. They used cluster analysis to identify groups of inbred strains with significant contributions to the heritability of GEI, and direct analysis of GEI effects on gene expression by employing replication of genomic expression assays within a subset of inbred strains representing the clusters associated with most of the relevant genetic variation.

Here we show that sets of RI strains can be used to identify QTL for developmental GEI effects similar to those described for olfactory behavior in non-recombinant inbred strains of flies raised on different food sources (Sambandan et al. 2008). Sambandan et al. (2008) also replicated gene expression assays within strains reared on different food sources and used analysis of variance to identify GEI at the level of transcription for individual loci. Future studies that include assays of global gene expression across RI lines will be able to directly correlate strain variation in expression with strain variation in complex trait phenotypes in different environments.

Developmental exposure to stressors (such as lead) can produce a range of behavioral changes in children, including a decrease in intelligence as measured by IQ tests (Bellinger 2008b; Jusko et al. 2008; White et al. 2007). We have suggested that lead is just one of many environmental factors that induces developmental changes (Hirsch and Ghiradella 2004) which may give rise to a slightly “modified” nervous system (Weiss and Bellinger 2006). This process of course involves changes in gene regulation (Bakheet et al. 2007;

Zawia et al. 2000) and a first step is to identify some of these genes. Given the conservative nature of evolutionary changes (Gaziova and Bhat 2007; Keshishian et al. 1996) genes identified in *Drosophila* may help identify those involved in the response to lead in children.

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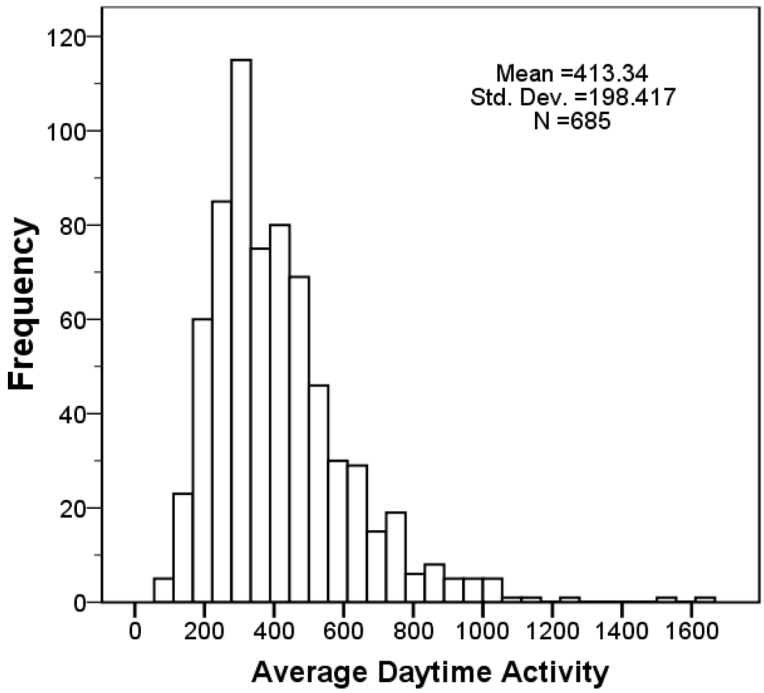

Figure 1.
Histogram of the distribution of ADA for control flies showing the variation in the behavioral trait (ADA) we used in our QTL analysis.

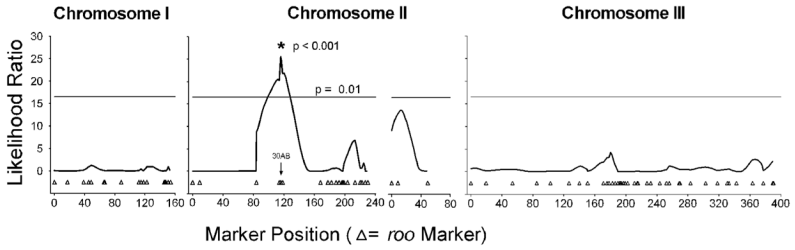

Figure 2.

Composite Interval Mapping for the ADA Lead Index trait showing a significant QTL at cytological marker 30AB The The Y axis is in Likelihood Ratio (LR) Units; the horizontal line indicates $p < 0.01$ significance level. The X axis is in centiMorgans (cM), representing the genetic map for chromosomes 1, 2 and 3.

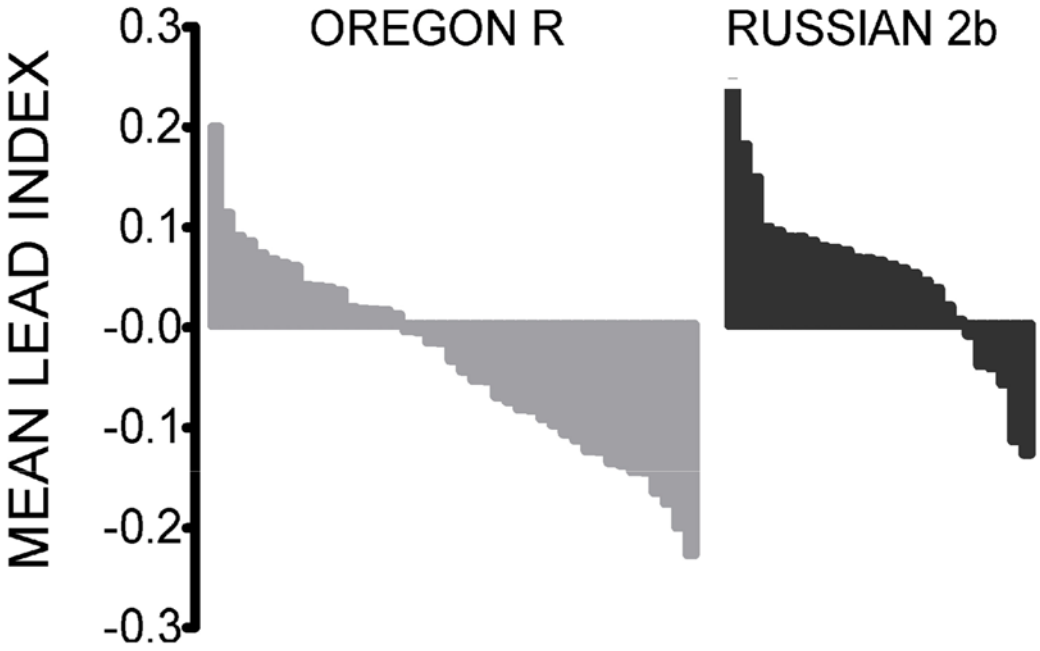

Figure 3.
Distribution of *roo* lines as a function of ADA Lead Index (ranked from largest to smallest) for flies in which 30AB is from the Oregon R parental line (left) versus the Russian 2b line (right). Note there are many more lines in which the index is negative (and thus lead reduces activity) for Oregon R than for Russian 2b.

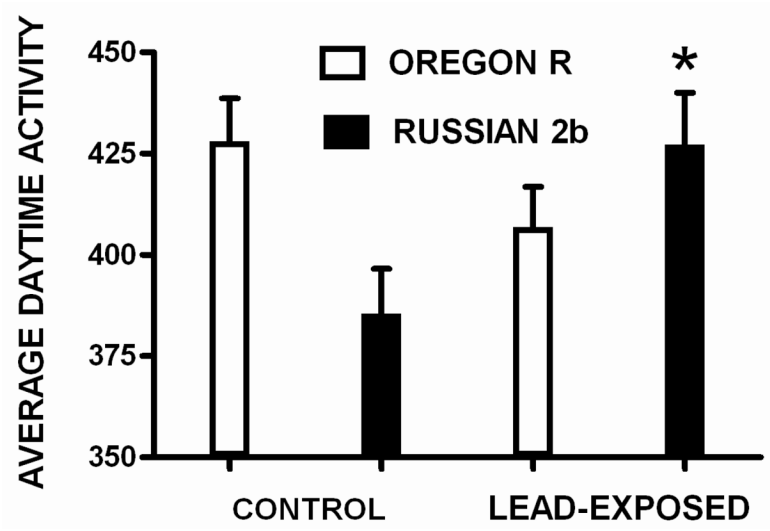

Figure 4.

ADA is graphed for flies representing only the Oregon R and Russian 2b parental genotypes at 30AB under control (left) and leaded (right) conditions. When the 30AB marker is Russian 2b, lead significantly increases ADA. When the 30AB marker is Oregon R, lead does not have a significant effect on ADA. The genotype by lead-treatment was significant ($p<0.005$). (Error bars indicate standard error of the mean; * = $p < 0.015$).

Table 1

Behavioral Results

Trait(n=1348)	Mean +/− SEM	ANOVAp-values for main effects and interactions[a]					
		lead	line	lead*line	rep	lead*rep	line*rep
Average Daily Activity	413.0 +/− 5.3	0.30	0.001	0.047	0.001	0.001	0.35
Average Nightly Activity	181.3 +/− 4.5	0.89	0.001	0.415	0.001	0.006	0.98
Average Total Activity	594.3 +/− 8.4	0.57	0.001	0.106	0.001	0.001	0.79

[a] p< value shown