Atmospheric hypoxia limits selection for large body size in insects

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Recent geological models indicate a marked increase in atmospheric oxygen partial pressure (aPO_2) to 32 kPa in the Permo-Carboniferous (≈300 million years ago), subsequently falling to 13 kPa in the Triassic[1]. These aPO_2 changes have been hypothesized to cause multiple major evolutionary events [2] including the appearance and subsequent extinction of giant insects and other taxa[3,4]. Patterns of increasing tracheal investment in larger insects support this hypothesis[5], as do observations of positive relationships between aPO_2 and body size in single- or multi-generational experiments with *Drosophila melanogaster* and other insects[6]. Large species likely result from many generations of selection for large body size driven by predation, competition or sexual selection[7]. Thus a crucial question is whether aPO_2 influences the capacity of such selection to increase insect size. We tested that possibility by selecting for large body size in five *Drosophila melanogaster* populations for 11 generations in hypoxic (10 kPa), normoxic (21 kPa) and hyperoxic (40 kPa) aPO_2, followed by three generations of normoxia without size selection to test for evolved responses. Average body sizes increased by 15% during 11 generations of size selection in 21 and 40 kPa aPO_2 flies and even stronger responses were observed for the flies in the largest quartile of body masses. However, flies selected for large size in 10 kPa aPO_2 had strongly reduced sizes compared to those in higher aPO_2. Upon return to normoxia, all flies had

similar, enlarged sizes relative to the starting populations. These results demonstrated that positive size selection had equivalent genetic effects on all flies independent of aPO_2, but that hypoxia provided a physical constraint on body size even in a relatively small insect under strong selection for larger mass. Our data support the hypothesis that Triassic hypoxia may have contributed to a reduction in insect size.

Limited multigenerational studies with *Drosophila melanogaster* suggest that these insects might evolve larger body sizes when aPO_2 is higher[8, 9]. However, body size can be affected by many factors, and it is not clear that interactions between oxygen and body size in the lab would occur in a similar manner in the field. Selection for large size, as often occurs in the wild, could potentially overcome these aPO_2 effects. *Drosophila melanogaster* exhibit strong changes in body size in response to artificial truncation selection for large size[10], and provide a convenient model for testing whether aPO_2 influences the response of a species to strong selection for larger body size.

To test this possibility, we performed truncation selection for 11 generations on five populations of *D. melanogaster* in 10, 21 and 40 kPa aPO_2 respectively. Each generation we selected the biggest 30 females and 20 males, representing approximately the largest 25% of each population, to found the next generation. After 11 generations the selection regime was lifted; and then a random 25% of each population was selected to found another three generations at 21 kPa aPO_2. At every generation we measured the body masses of randomly selected males (n = 20) and females (n = 30) for each population. In addition, because prior research suggests that oxygen may have stronger effects on maximal compared to mean size[11,12], we also measured the masses of the flies selected to found the next generations (the largest quartile).

During size selection, both mean masses and largest quartile masses of flies reared in 21 or 40 kPa aPO_2 showed marked increases (Figs. 1, 2 and Table 1). After 11 generations, for the five populations of flies selected in 21 or 40 kPa aPO_2, mean mass increased significantly by 11-17% over generation 0 values, and the upper quartile sizes increased by 25-32 %. In most cases, there were no significant size differences between the 21 and 40 kPa groups (see Figs. 1, 2 and aPO_2 effects in Table 1). By contrast, the flies selected for large size in 10 kPa aPO_2 decreased in size during the initial selection generations (Fig. 1). After 11 generations of selection, the mean size of the five populations reared in 10 kPa aPO_2 did increase but did not differ significantly from the starting populations (Fig. 2). Size selection increased the upper quartile sizes of the flies reared in 10 kPa by 5-8%. Nevertheless, the sizes of all flies reared in 10 kPa aPO_2 remained well below those of flies reared in 21 kPa or 40 kPa aPO_2 throughout the selection period (see Figs. 1, 2 and aPO_2 effects in Table 1).

When the populations were returned to normoxia (and random mating), the masses of the groups reared in the three different aPO_2s converged within one generation toward the greater masses attained by the 21 and 40 kPa groups. Regardless of prior aPO_2, the populations' mean increase in mass relative to generation 0 was 2-11%, while the largest quartile flies increased in size by 12-21%. Clearly truncation selection successfully changed both the mean values and the size distribution of these populations. The similarity of the masses of the groups in generations 12-14 indicates that the selection-induced genetic changes related to size were similar and independent of historical aPO_2 during selection.

Our data did not support the hypothesis that atmospheric hyperoxia would enable the evolution of larger insects in a strong size selective environment, as hyperoxic rearing did not allow flies to reach larger sizes relative to normoxic rearing. In general, phenotypic plastic responses of *D. melanogaster* body size to 40 kPa aPO_2 are relatively

small (3-6%)[13] and it is not surprising that selection can overcome such a minor plastic effect. Conceivably, a different result would occur at a less extreme level of hyperoxia. Forty kPa aPO_2 is near the highest level of oxygen for successful rearing of some *D. melanogaster* strains[14], and thus at this aPO_2 there may be oxidative stress that counters positive effects of hyperoxia on size. Also, one should take into account that *D. melanogaster* is a very small insect, and potentially the interactions between body size and oxygen delivery might differ in much larger insects, such as the giant Palaeozoic palaeopterans. The correlations between increased aPO_2 during this era[1,2] and insect gigantism[2, 3,4], as well as experimental evidence of increased body size of insects reared in hyperoxia[6] lend support to the hypothesis that atmospheric hyperoxia contributed to the evolution of gigantism.

By contrast, this study's data convincingly show that hypoxia can limit the size of insects, even when they are strongly selected for large size (Fig. 1). Is it reasonable to extrapolate from the small *D. melanogaster* to the giant insects of the Palaeozoic? Hypoxia suppresses size in most of the modern insects that have been studied, at least in single generation studies[6]. These plastic effects of hypoxia on size in *D. melanogaster* are possibly mediated via oxygen-dependent signalling pathways regulating growth and developmental processes such as the ISS pathway (Insulin/Insulin like growth factor signalling glucose transport and cell growth), IDGFs (chitinase related imaginal disc growth factors), ADGFD (adenosine-deaminase related growth factor),[15] HIF-1α (hypoxia inducible factor)[16,17], or via Tuberous Sclerosus Complex 2 (Tsc2) or Redd1-mediated suppression of TOR signalling[18,19]. Analogous representatives of these signalling pathways have been characterized in *Hydra* (Coelenterata)[20], *Caenorhabditis elegans* (Nematoda)[21,22], *Daphnia magna* (Crustacea)[23], *D. melanogaster* (Insecta)[15,23], various mammals[24], yeast and *Arabidopsis*[25]. This broad distribution of oxygen-dependent growth among organisms indicates that these signalling pathways originated in their common ancestry at least 500 million years ago[25], are highly conserved among

eukaryotes, and therefore likely also regulated the development of the Palaeozoic giant insect species such as *Meganeura monyi* and *Meganeuropsis permiana* (Order Protodonata)[26] and *Mazothairos enormis* (Order Palaeodictyoptera)[27]. Thus, our data, demonstrating strong size suppression in a small insect selected for large size, indicates that decreased aP_{O_2} offers an important explanation for the giant palaeopteran species' extinction during the progressively hypoxic aP_{O_2} across the Permo-Triassic boundary[1].

Methods

To maximize genetic diversity, starting populations were derived by outbreeding five unrelated *Drosophila melanogaster* lines (Tucson *Drosophila* Stock Center numbers: 14021-0231.20, 14021-0231.24, 14021-0231.35, 14021-0231.38, 14021-0231.43). Outbred stocks were treated with tetracycline and rifampicin (3-5 generations) to eliminate *Wolbachia* [28,29]. Two antibiotic-free generations preceded selection experiments, and the experimental media lacked antibiotics.

Generation 0. We split our outbred stock into 15 populations (5 replicates per aP_{O_2}, each started with 30♀ and 20♂. <48 hours old). Flies were cold-anaesthetized (1hr at 4±1°C)[30], weighed individually (Mettler MX 5, ±0.001 mg; and placed in 237 ml bottles with 50 ml standard yeast-based *Drosophila* growth medium. Bottles were kept in an incubator (Percival, Boone IO, 25°C, 12L:12D photoperiod) inside three air-tight chambers, each connected to a Sable Systems ROXY-8 paramagnetic oxygen regulation system that regulated aP_{O_2} at 10, 21 and 40 kPa (www.sablesys.com/roxy8.html). Adult flies were removed after four days to limit larval densities to <250/bottle.

Size selection - Generations 1 to 11. To determine mean population masses, we weighed haphazardly-chosen 30♀ and 20♂ per population. Of these, the heaviest 10♀ and 6♂ were placed in new bottles and served as a portion of the founders of the next

generation. From the other flies, we visually selected and individually weighed the largest 35♀ and 25♂. Preliminary analyses confirmed that we could visually select flies whose average mass did not differ significantly from actual largest masses, ANOVA: $F_{4, 45} = 0.619$, $p = 0.65$. The heaviest 20 out of the 35♀ and 14 out of the 25♂ comprised the remaining founders of the next generations.

For generations 12-14, selection ceased and populations were reared at 21 kPa. Randomly selected adults (30♀ and 20♂) founded each generation, and we continued to measure mean and largest upper quartile masses as described above.

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Table 1. Comparisons of fly sizes at the start vs the end of positive size selection. Repeated measures ANOVA statistics for the first and last generations that experienced directional selection for larger size, comparing hypoxic-reared (10 kPa, top) or hyperoxic-reared flies (40 kPa, bottom) to the control or normoxic-reared flies (21 kPa). Significant p values are boldfaced. In all cases, hypoxic-reared flies were significantly smaller than normoxic-reared flies, and responded differently than normoxic-reared flies. 10 kPa flies had a lesser increase in mass with size selection, indicated by significant aPO_2 x Generation terms.

	Mean sizes			Upper quartile sizes		
Effect	F	DF	p	F	DF	p
10 kPa vs 21 kPa: Generations 1 vs 11, during truncation selection for large size						
	Females			Females		
aPO_2	69.09	2, 15	**<0.0001**	89.75	2, 15	**<0.0001**
Generation	95.98	2, 15	**<0.0001**	77.98	2, 15	**<0.0001**
aPO_2 x Generation	23.28	2, 15	**<0.0001**	24.07	2, 15	**<0.0001**
	Males					
aPO_2	45.32	2, 15	**<0.0001**	95.52	2, 15	**<0.0001**
Generation	39.52	2, 15	**<0.0001**	157.58	2, 15	**<0.0001**
aPO_2 x Generation	9.18	2, 15	**<0.0025**	14.18	2, 15	**<0.0004**
21 kPa vs 40 kPa: Generations 1 vs 11, during truncation selection for large size						
	Females			Females		
aPO_2	0.05	2, 15	0.9531	4.36	2, 15	**<0.0322**

Generation	52.14	2, 15	**<0.0001**	36.20	2, 15	**<0.0001**
aPO_2 x Generation	3.04	2, 15	0.0781	1.52	2, 15	0.2500
	Males			Males		
aPO_2	0.921	2, 15	0.4197	0.71	2, 15	0.5084
Generation	73.46	2, 15	**<0.0001**	62.90	2, 15	**<0.0001**
aPO_2 x Generation	7.23	2, 15	**<0.0063**	3.33	2, 15	0.0636

Table 2. Comparisons of initial fly sizes vs. post selection fly sizes. Repeated Measures ANOVA statistics (α = 0.05) for the starting populations at Generation 0 vs the second generation (Generation 13) of populations post-size selection and returned to normoxia. Although all these flies were reared in normoxia, the analyses compare hypoxic-selected (10 kPa, top) or hyperoxic-selected flies (40 kPa, bottom) to control or normoxic-selected flies (21 kPa). Significant p values are boldfaced. In general, flies were larger in generation 13 than in the starting populations, indicating evolution of larger size in response to truncation selection (significant generation effects). However, in general, there were no significant effects of the aPO_2 during the period of size selection.

	Mean sizes			Upper quartile sizes		
Effect	F	DF	p	F	DF	p
10 kPa vs 21 kPa: Generations 0 pre- vs 13 post-size selection						
	Females			Females		
aPO_2	1.06	2, 15	0.3722	0.91	2, 15	0.4222
Generation	3.81	2, 15	**<0.0459**	20.58	2, 15	**<0.0001**
aPO_2 x Generation	0.17	2, 15	0.8430	0.52	2, 15	0.6062
	Males			Males		
aPO_2	3.55	2, 15	0.0545	1.43	2, 15	0.2713
Generation	7.89	2, 15	**<0.0045**	24.29	2, 15	**<0.0001**
aPO_2 x Generation	0.02	2, 15	0.9778	0.20	2, 15	0.8252
21 kPa vs 40 kPa: Generations 0 pre- vs 13 post-size selection						
	Females			Females		
aPO_2	0.31	2, 15	0.7354	1.42	2, 15	0.2715

Generation	1.38	2, 15	0.2826	24.82	2, 15	**<0.0001**
aPO_2 x Generation	0.52	2, 15	0.6037	0.16	2, 15	0.8570
	Males			Males		
aPO_2	2.82	2, 15	0.0915	2.35	2, 15	0.1292
Generation	13.19	2, 15	**<0.0005**	35.46	2, 15	**<0.0001**
aPO_2 x Generation	10.89	2, 15	**<0.0012**	14.80	2, 15	**<0.0003**

Figure legends

Figure 1. *Drosophila melanogaster* specimens (females left, males right) from the large size-selected populations maintained in their test aPO_2s. The flies in 21 and 40 kPa had very similar body sizes but those maintained in 10 kPa exhibited strong size suppression despite having undergone strong size selection for 11 generations.

Figure 2. Plots of mass changes across generations. Mean adult masses (females above, males below) of five selected populations of *Drosophila melanogaster* (left), and mean masses of the largest quartile of those populations (values shown are the means ± 0.95 confidence intervals of the five population means for each treatment). Generation zero represents initial values of starting populations all reared in 21 kPa (included in red box). From generations 1-11, directional selection for large size was applied in either hypoxic (10 kPa, ●), normoxic (21 kPa, ■) or hyperoxic (40 kPa, ◆) conditions. During generations 12-14, populations were returned to 21 kPa (included in red box) and no selection was performed. In all cases, across all generations, Repeated Measures ANOVAs ($\alpha = 0.05$) showed that the aPO_2 and Generation effects and the aPO_2 x Generation interactions were highly significant ($p < 0.0001$). Non-overlapping 0.95 CI whiskers indicate significant differences. Due to questionable growth medium quality, generations 5, 8 and 9 were excluded from all analyses.

Figure 1.

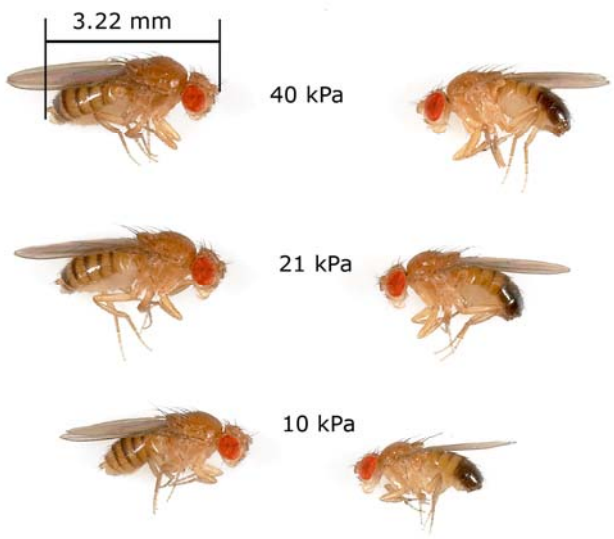
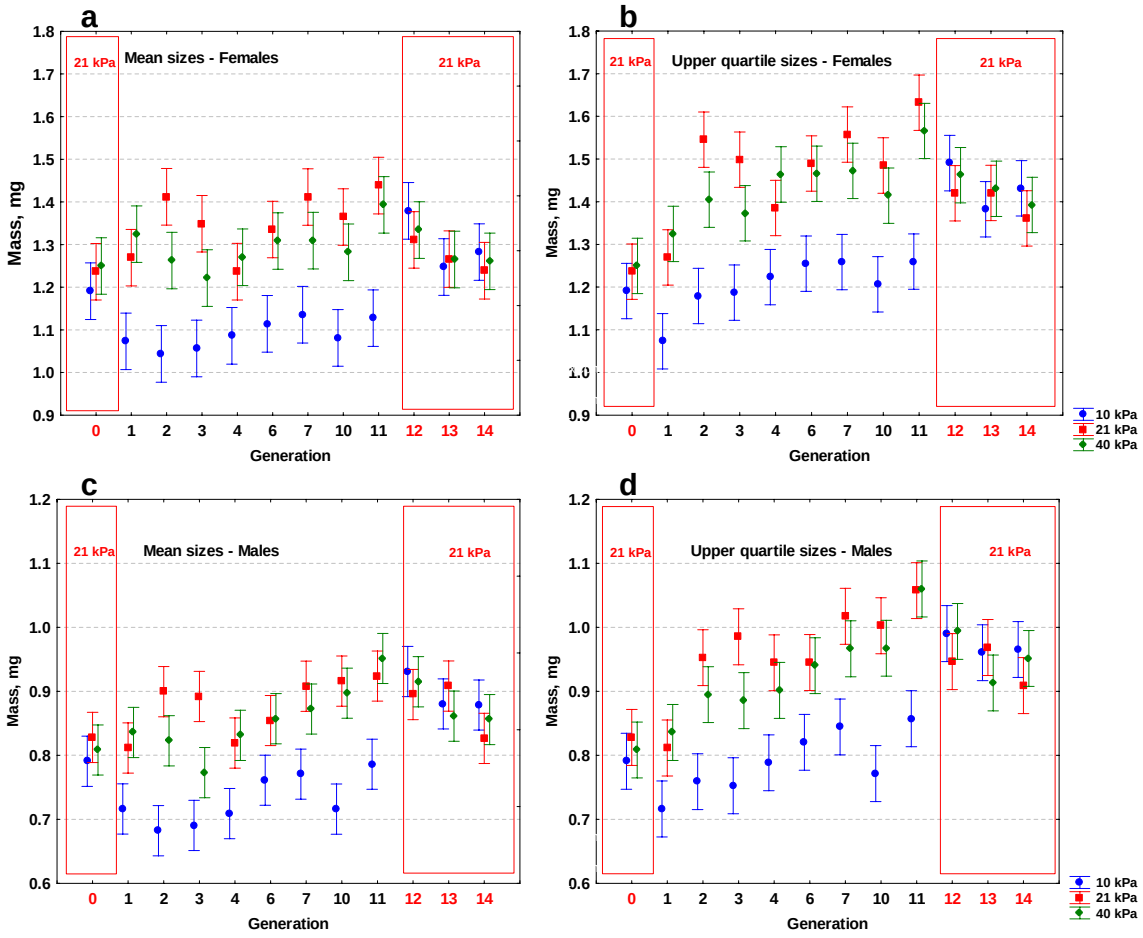


Figure 2


a
Mean sizes - Females
21 kPa
21 kPa
Mass, mg
Generation
b
Upper quartile sizes - Females
21 kPa
21 kPa
Mass, mg
Generation
c
Mean sizes - Males
21 kPa
21 kPa
Mass, mg
Generation
d
Upper quartile sizes - Males
21 kPa
21 kPa
Mass, mg
Generation
10 kPa
21 kPa
40 kPa