Atmospheric Hypoxia Limits Selection for Large Body Size in Insects

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Abstract

Background: The correlations between Phanerozoic atmospheric oxygen fluctuations and insect body size suggest that higher oxygen levels facilitate the evolution of larger size in insects.

Methods and Principal Findings: Testing this hypothesis we selected Drosophila melanogaster for large size in three oxygen atmospheric partial pressures (aPO2). Fly body sizes increased by 15% during 11 generations of size selection in 21 and 40 kPa aPO2. However, in 10 kPa aPO2, sizes were strongly reduced. Beginning at the 12th generation, flies were returned to normoxia. All flies had similar, enlarged sizes relative to the starting populations, demonstrating that selection for large size had functionally equivalent genetic effects on size that were independent of aPO2.

Significance: Hypoxia provided a physical constraint on body size even in a tiny insect strongly selected for larger mass, supporting the hypothesis that Triassic hypoxia may have contributed to a reduction in insect size.

Introduction

Recent geological models indicate a marked increase in atmospheric oxygen partial pressure (aPO2) to 32 kPa in the Permo-Carboniferous (~300 million years ago), subsequently falling to 13 kPa in the Triassic [1]. These atmospheric oxygen partial pressure (aPO2) 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 aPO2 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 aPO2 influences the capacity of such selection to increase insect size. We tested that possibility by subjecting Drosophila melanogaster populations to truncation selection for large size for 11 generations in hypoxic (10 kPa), normoxic (21 kPa) and hyperoxic (40 kPa) aPO2, followed by three generations of normoxia without size selection.

Limited multigenerational studies with Drosophila melanogaster suggest that these insects might evolve larger body sizes when aPO2 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. Drosophila melanogaster exhibits strong changes in body size in response to artificial truncation selection for large size [10], and provide a convenient model for testing whether aPO2 influences the response of a species to strong selection for larger body size.

Results

During size selection, we measured both mean population masses and also the masses of the largest quartile of flies, which were the flies selected to found generations 2 to 11. Both mean population masses and largest quartile masses of flies reared in 21 or 40 kPa aPO2 showed marked increases in response to size selection (Figs. 1, 2 and Table 1). After 11 generations, for the five populations of flies selected in 21 or 40 kPa aPO2, 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 aPO2 effects in Table 1). By contrast, the flies selected for large size in 10 kPa aPO2 decreased in size during the initial selection generations, and then slowly increased (Fig. 1). After 11 generations of selection, the mean size of the five populations reared in 10 kPa aPO2 did not differ significantly from the starting populations (Fig. 2). Size selection significantly increased the upper quartile sizes of the flies reared in 10 kPa by 5–8% relative to the starting populations. Nevertheless, the sizes of all flies reared in 10 kPa aPO2 remained well below those of flies reared in 21 kPa or 40 kPa aPO2 throughout the selection period (see Figs. 1, 2 and aPO2 effects in Table 1).

When the populations were returned to normoxia (and random mating), the masses of the groups reared previously in the three different aPO2s converged within one generation toward the greater masses attained by the 21 and 40 kPa groups. Regardless of prior aPO2, the populations’ mean increase in mass relative to generation 0 was 2–11%, while the largest quartile flies increased in size by 12–21% (Table 2). Clearly truncation selection
larger population sizes and more generations, that the hypoxic-reared flies could attain the size of flies selected in normoxia. However, the trends in our experiments suggest the alternative, that greater populations and time would increase the divergence induced by aPO2 (Fig. 2).

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) [14], HIF-1α (hypoxia inducible factor) [15,16], or via Tuberous Sclerosus Complex 2 (Tsc2) or Redd1-mediated suppression of TOR signalling [17,18]. Analogous representatives of these signalling pathways have been characterized in Hydra (Coelenterata) [19], Caenorhabditis elegans (Nematoda) [20,21], Daphnia magna (Crustacea) [22], D. melanogaster (Insecta) [14,22], various mammals [23], yeast and Arabidopsis [24]. This broad distribution of oxygen-dependent growth among organisms indicates that these signalling pathways originated in their common ancestry at least 300 million years ago [24], are highly conserved among eukaryotes, and therefore likely also regulated the development of the Palaeozoic giant insect species such as Meganeura monyi and Meganeura permaina (Order Protodonata) [25] and Mazoikarios enormis (Order Palaeodictyoptera) [26]. Thus, our data, demonstrating strong size suppression in a small insect selected for large size, strongly supports the hypothesis that decreased aPO2 could explain the giant palaeopteran species’ extinction during the progressively hypoxic aPO2 across the Permo-Triassic boundary [1].

Materials and Methods

To test this potential effect of atmospheric oxygen concentration on positive size selection, we performed truncation selection for 11 generations on five populations of D. melanogaster in 10, 21 and 40 kPa aPO2 respectively. 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, 14021-0231.30, 14021-0231.43). As a precaution to unpredictable events during selection, these outbred stocks were treated with tetracycline and rifampicin for 3–5 generations prior to the start of truncation selection procedures to eliminate Wolbachia infections [27,28]. 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 aPO2, each started with 30Q and 20Q newly eclosed flies, <48 hours old). The flies were cold-anesthetized (1 hr at 4 ± 1°C) [29], weighed individually (Mettler MX 5, ±0.001 mg), and placed in 237 ml bottles with 50 ml standard yeast-based Drosophila growth medium. The 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 aPO2 at 10, 21 and 40 kPa (www.sablesys.com/roxy8.html). Adult flies were allowed to mate randomly and oviposit for four days after which they were removed to limit larval densities to <250/bottle.

Discussion

Our data did not support the hypothesis that atmospheric hypoxia would enable the evolution of larger insects in a strong size selective environment, as hypoxic 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 aPO2 are relatively small (3–6%) [11] 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 hypoxia. Forty kPa aPO2 is near the highest level of oxygen for successful rearing of some D. melanogaster strains [12], and thus at this aPO2 there may be oxidative stress that counters positive effects of hypoxia on size. However, it has also been demonstrated that insects can control their spiracular openings to limit the potentially detrimental effect of too much oxygen [13]. Additionally, with larger or different populations, and more variance available for selection, it is possible that hypoxia might affect responses to selection. 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 aPO2 during this era [1,2] and insect gigantism [2–4], as well as experimental evidence of increased body size of insects reared in hyperoxia [6] lend support to the hypothesis that atmospheric hypoxia 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). We cannot exclude the possibility that with
To determine mean population masses, we weighed 30 and 20 newly eclosed adult flies (haphazardly-chosen) per population. Of these, the largest 10 and 6 per population 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 in each population, ANOVA: F4, 45 = 0.619, p = 0.65. These visually selected 35 and 25 were then weighed individually and sorted according to mass. From these, the largest 20 and 14 were added to the largest 10 and 6 mentioned above. This additional procedure ensured that we selected flies from the actual largest quartile of the population. Together these size-selected 30 and 20 adults founded 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, because prior research suggests that the effects of oxygen may be stronger on maximum sizes compared to mean sizes [30,31].

Statistical analyses

Data sets for ‘mean population masses’ and ‘upper quartile masses’ were compiled and analyzed separately using STATISTICA 8 (www.StatSoft.com). Females and males were analyzed separately. At each generation, the mean masses of each sex for each population and the mean mass of the largest quartile of flies for each sex and population were used as data, giving an n = 5 for each selection group. A repeated measures ANOVA design
Table 1. Statistical analyses of fly size variation at the start vs the end of positive size selection.

| Effect | Population mean sizes | Upper quartile sizes |
|--------|------------------------|----------------------|
|        | F | DF | p      | F | DF | p      |
| 10 kPa vs 21 kPa: Generations 1 vs 11, during truncation selection for large size | | | | | | |
| Females | | | | | | |
| aPO2 | 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 |
| aPO2 x Generation | 23.28 | 2, 15 | <0.0001 | 24.07 | 2, 15 | <0.0001 |
| Males | | | | | | |
| aPO2 | 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 |
| aPO2 x Generation | 9.18 | 2, 15 | <0.0001 | 14.18 | 2, 15 | <0.0001 |
| 21 kPa vs 40 kPa: Generations 1 vs 11, during truncation selection for large size | | | | | | |
| Females | | | | | | |
| aPO2 | 0.05 | 2, 15 | 0.9531 | 4.36 | 2, 15 | <0.0001 |
| Generation | 52.14 | 2, 15 | <0.0001 | 36.20 | 2, 15 | <0.0001 |
| aPO2 x Generation | 3.04 | 2, 15 | 0.0781 | 1.52 | 2, 15 | 0.2500 |
| Males | | | | | | |
| aPO2 | 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 |
| aPO2 x Generation | 7.23 | 2, 15 | <0.0001 | 3.33 | 2, 15 | 0.0636 |

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 aPO2 x Generation terms. (F = F-ratio; DF = degrees of freedom).

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Table 2. Statistical analyses of variation of initial fly sizes vs. the size of flies post-selection—all reared in normoxic conditions.

| Effect | Population mean sizes | Upper quartile sizes |
|--------|------------------------|----------------------|
|        | F | DF | p      | F | DF | p      |
| 10 kPa vs 21 kPa: Generations 0 pre- vs 13 post-size selection | | | | | | |
| Females | | | | | | |
| aPO2 | 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 |
| aPO2 x Generation | 0.17 | 2, 15 | 0.8430 | 0.52 | 2, 15 | 0.6062 |
| Males | | | | | | |
| aPO2 | 3.55 | 2, 15 | 0.0545 | 0.71 | 2, 15 | 0.5084 |
| Generation | 7.89 | 2, 15 | <0.0045 | 24.29 | 2, 15 | <0.0001 |
| aPO2 x Generation | 0.02 | 2, 15 | 0.9778 | 0.02 | 2, 15 | 0.8252 |
| 21 kPa vs 40 kPa: Generations 0 pre- vs 13 post-size selection | | | | | | |
| Females | | | | | | |
| aPO2 | 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 |
| aPO2 x Generation | 0.52 | 2, 15 | 0.6037 | 0.16 | 2, 15 | 0.8570 |
| Males | | | | | | |
| aPO2 | 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 |
| aPO2 x Generation | 10.89 | 2, 15 | <0.0012 | 14.80 | 2, 15 | <0.0003 |

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 previously hypoxic-selected (10 kPa, top) or previously hyperoxic-selected flies (40 kPa, bottom) to control flies that experienced size selection in normoxia (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, there were no significant effects of the aPO2 during the period of size selection. (F = F-ratio; DF = degrees of freedom).

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tracked the changes in size across generations for each oxygen concentration.

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**Author Contributions**

Conceived and designed the experiments: JH. Performed the experiments: CJK. Analyzed the data: CJK. Wrote the paper: CJK. Modified the experiment: CJK. Contributed to interpretation of the analyzed data: JH.

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