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Drosophila melanogaster locomotion in cold thin air

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Summary

The alpine environment is likely to challenge insect locomotion because of low mean temperatures and reduced barometric pressure. In this study, we measured the direct and interactive effects of these factors on walking and flight performance of wild-caught *Drosophila melanogaster* Meigen. We found that decreased temperature and decreased air pressure both reduced walking speed and flight performance. Flies walked more slowly at 18°C and in the lowest air pressure treatment (34 kPa). This treatment, equivalent in air pressure to the top of Mount Everest, was the only air pressure that significantly reduced fly walking speed. Therefore, walking performance in the wild is likely limited by temperature, but not oxygen availability. In contrast to walking performance, low but ecologically realistic air pressures dramatically reduced overall flight performance. The effects of reduced air pressure on flight performance were more pronounced at colder temperatures. Reduced flight performance in high altitude conditions was primarily driven by an increased reluctance for flies to initiate flight rather than outright failure to fly. Such reluctance to fly in high altitude conditions may in part explain the prevalence of aptery and brachyptery in high altitude insects. The observed interactive effects of temperature and air pressure on flight performance confirm the importance of simultaneously manipulating both of these factors when studying the impact of altitudinal conditions on insect physiology and behavior.

Key words: *Drosophila melanogaster*, high altitude, mountain, flight, walking speed, temperature, physiology, interaction, air density, air pressure, oxygen.

Introduction

Altitudinal gradients are characterized by rapid changes in the physical environment. Mean air temperature drops by 6°C km^{-1}, such that the temperature change in climbing from sea level to 4000 m is roughly equivalent to the change experienced in traveling 4500 km in latitude (Dillon et al., in press). Accompanying this rapid temperature change, at 4000 m the partial pressure of oxygen (P_{O_2}) falls to half its sea level value, and air density decreases by 40%. These physical changes should, both individually and in concert, compromise the physiology of ectothermic insects (Mani, 1968; Sømme, 1989, 1995). Despite these challenges, however, many ectothermic insects maintain robust populations on mountains (e.g. Mani, 1968; Brehm et al., 2003; Romero-Alcaraz and Avila, 2000). Fruit flies in the genus *Drosophila* (family *Drosophilidae*) are found from less than 100 m to over 3000 m in the Californian Sierra Nevada (Dobzhansky, 1948) and above 5000 m in the Indian Himalayas (Khare et al., 2002). To maintain robust populations on high mountains, these small ectothermic insects must locate food and mates, and evade predators, despite the combined physiological challenges of low temperature, low P_{O_2} and low air density. Here we ask whether these factors, independently and in concert, compromise insect locomotory performance.

Decreased temperatures at high altitude affect insect performance because physiological reaction rates are strongly determined by temperature (Huey and Kingsolver, 1989). Low temperatures reduce metabolic rates and change the dynamics of muscle contraction (Josephson, 1981), impairing muscle physiology (Scaraffia and De Burgos, 2000; Hosler et al., 2000) and therefore locomotory performance of fruit flies. Walking speed of *D. melanogaster* is slower in colder temperatures (Gibert et al., 2001; Crill et al., 1996). Similarly, in tethered flies, less power is produced for flight at low temperatures (Curtsinger and Laurie-Ahlberg, 1981; Lehmann, 1999).

Reduced P_{O_2} may compromise fruit fly locomotion by reducing metabolic rate. The dramatic altitudinal reduction in P_{O_2} may challenge tissue oxygen delivery because insects depend on diffusion (at least through the terminal tracheoles) for which P_{O_2} is the driving force (Denny, 1993). However, oxygen delivery may not limit insects at high altitude because insects are generally very resistant to low oxygen levels (Krishnan et al., 1997; Hoback and Stanley, 2001; but see below for potential interactions

between P_{O_2} and temperature and their effects on oxygen delivery).

Reduced air density should make it difficult to fly at high altitude because aerodynamic forces produced by wing flapping increase linearly with air density (Dudley, 2000). Despite this theoretical limitation, many insects fly on high mountains (Mani, 1968). Furthermore, in the laboratory, orchid bees fly in pure heliox (21% O_2, balance helium), despite the 64% reduction in air density (approximately equivalent to the top of Mount Everest; Dudley, 1995). To do so, orchid bees increase mechanical power output by 40–50%, suggesting that flight in low air densities, though possible, may be energetically expensive.

Some research exists on the independent effects of temperature, P_{O_2}, and air density on insect locomotory performance. However, high altitude ecosystems are uniquely characterized by the combination of these factors, yet no study has investigated the interactions among these factors and their potential synergistic effects on insect locomotion. Recent work documenting large interactive effects of temperature and oxygen on insect development (Frazier et al., 2001; Woods and Hill, 2004) strongly suggests that such interactions should be considered in studies of insect physiology.

Here we measure the direct and interactive effects of temperature and barometric pressure on walking and flight performance of wild-caught *Drosophila melanogaster*. We use a standard technique to measure walking speed (Crill et al., 1996; Gilchrist, 1996) and introduce a novel method to measure whole animal flight ability and flight motivation. We find strong negative effects of reduced temperature and reduced air pressure on flight ability and walking speed. Low temperatures and low air pressures interact to reduce flight performance more than predicted by the additive effects of these variables. Our results suggest that future studies on high altitude physiology incorporate the suite of physical factors that characterize high altitude ecosystems.

Materials and methods

Experimental animals

In July 2001, we collected female *D. melanogaster* Meigen from a pear orchard near Wenatchee, WA, USA (47°34′N, 120°36′W). We combined the 38 resulting isofemale lines into a single colony, which served as the source of our experimental flies. To maintain the colony, we collected eggs approximately every 10 days and reared them in controlled conditions (50 eggs/vial; ~23°C and ~16 h:8 h L:D; diet of cornmeal, molasses, yeast, agar, tegosept) before transferring newly emerged adult flies into the colony. Colony food bottles (150 ml) were replaced every 3–5 days, ensuring that all colony flies developed in controlled conditions. The colony was maintained at 1000–1500 flies for 3–6 generations prior to experiments.

Experimental flies were collected as eggs during a 2–6·h laying period and reared at densities of 50 eggs/vial. To control for age and reproductive state, we transferred newly emerged female flies to fresh food vials every 24·h and allowed the flies to mature for 3–4 days before testing their locomotory performance (we only tested virgin flies). We chose this age because wing-beat frequency and power output of flies remains constant from 2 to 8 days of age (Curtsinger and Laurie-Ahlberg, 1981). To assess performance, we first starved flies for 16–20·h in individual 1.5·ml Eppendorf tubes containing fresh agar for water; moderate starvation increases average flight speed (David, 1978) and duration (Tammero and Dickinson, 2002), and preliminary experiments demonstrated that starvation motivated flight. Then, individual flies were randomly assigned to a single temperature/air pressure treatment to test either walking or flight performance. Experiments were performed in a walk-in environmental chamber; humidity was kept near 100%, and temperature was monitored using a calibrated thermocouple thermometer (Physitemp Bat–12, Bailey Instruments Inc., Saddlebrook, NJ, USA). Given the relationship between air density, pressure and temperature, we could not simultaneously keep both air pressure and air density constant at different temperatures. We chose to keep air density constant because of its direct link to production of flight forces; consequently, at a given air density, air pressure increases slightly with temperature.

Walking performance

We modified the technique of Gilchrist et al. (1997) to assess walking speed of flies at three temperatures and four air densities: 18, 25, 30°C (18.09±0.38°C, 25.34±0.18°C and 29.61±0.33°C, means ± s.d., respectively); 33%, 50%, 66%, and 100% sea-level air pressure (34.27±0.74 kPa, 51.19±0.68 kPa, 67.06±1.04 kPa and 100.97±1.75 kPa, means ± s.d., respectively). We placed 10 flies individually inside 10 ml (6 ml volume after we had cut them down) plastic graduated burets that were connected in series to a vacuum pump. After adjusting air pressure inside the burets, we allowed the flies to acclimate for 5 min. We then knocked each fly to the bottom of its buret and timed to the nearest 0.01 s how long it took to walk 12 cm vertically. This was repeated twice more for each fly and the average was used to calculate walking speed. If a fly jumped, flew or walked in a spiral, the time was discarded and the fly was knocked to the bottom of the buret and retimed.

Flight performance

We assessed flight performance at four temperatures and at four air densities: 18, 25, 30, 32°C (18.05±0.23, 25.22±0.51, 30.11±0.28, 31.91±0.28, mean ± s.d., respectively); 33%, 50%, 66% and 100% sea level (33.28±1.81 kPa, 50.87±1.34 kPa, 67.46±1.22 kPa and 101.82±1.6 kPa, means ± s.d., respectively). The flight chambers were 250 ml glass milk bottles inverted over rubber stoppers to form an air-tight seal (Fig. 1). The interior of the bottle was coated with a fluon line that extended from the lowermost point of the bottle to 2 cm above the lip of the Eppendorf tube housing the fly (see Fig. 1). Flies were unable to walk up the fluon-coated sides of the bottle and thus could only arrive above the fluon line by flying. We

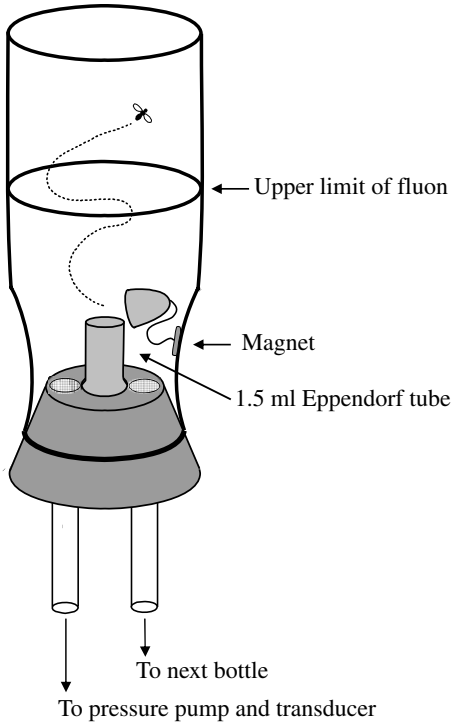

Fig. 1. Experimental setup for assessing flight ability of *D. melanogaster*. Ten bottles were connected in series to test ten flies simultaneously. Flies were held for 5 min in the Eppendorf tube to acclimate to the treatment. The lid of the tube was then removed with a magnet, releasing the fly into the chamber. We ranked flight performance as one of three categories: flew above the fluon line within 2 min ('flight'), flew within an additional 2 min period while the bottle was gently tapped ('coerced flight'), or failed to fly ('no flight').

arranged ten flight chambers in series, and used a vacuum pump to alter air pressure inside all of the chambers simultaneously. To motivate flight, we placed lights above the flight chambers and dabbed a small amount of apple cider vinegar at the top of each flight chamber before each test.

After a 16–20 h starvation period in individual Eppendorf tubes, the flies, while still housed in their starvation tubes, were individually placed in the flight chambers (Fig. 1). Pressure was then adjusted and the flies were given 5 min to acclimate to the treatment condition. After this acclimation period, the flies were released by removing the tube lids with a magnet (see Fig. 1).

We scored flight performance as follows: if a fly flew above the fluon line within 2 min after being released into the bottle, it was scored as a 'flight'. In pilot experiments at 25°C and sea level air density, almost all flies (>95%) flew within this 2 min period. For those flies that did not fly in the first 2 min, we gently tapped their bottles to encourage flight. Flies that flew above the fluon line during this 2 min agitation period were scored as 'coerced flight'. If a fly did not fly during the agitation period, we returned the bottle to sea-level air pressure. If the fly did not fly during 5 min of agitation at sea-level air pressure, we assumed it was somehow compromised and removed it from the analysis. However, if a fly flew during this sea-level agitation period, it was scored as 'no flight'. Therefore, those flies that failed to fly did so because of treatment conditions, not because they were incapable of flight.

Immediately after assessing flight performance, we weighed flies to the nearest 0.001 mg (Cahn C-33 microbalance, Cahn Instruments, Inc., Cerritos, CA, USA) and placed them in 95% ethanol. We later removed one wing from each fly and mounted it on a microsope slide (using Aquamount; VWR, Pittsburgh, PA, USA). We took digital pictures (Nikon Coolpix 990, Nikon Corporation, Tokyo, Japan) of fly wings through a microscope (Nikon, Japan) and determined area of the wing using a custom image analysis program (G. Wang: *WingWang: utilities for wing morphology analysis*, 2004; http://students.washington.edu/gw0/matlabcode). Wing loading was calculated as the ratio of fly weight to twice the measured area of one wing.

Statistical analyses

We assessed the effects of temperature and air pressure on walking speed using a full-factorial analysis of variance (ANOVA) with temperature and air pressure as factors. We used Tukey Honest Significant Difference (HSD) tests for *post-hoc* comparisons. To estimate statistical power we used a randomization technique (Fisher, 1935; Crawley, 2002). We simulated data by sampling from normal distributions with means and standard deviations (s.d.) set to the observed values of each treatment group and performed an ANOVA on the simulated data. We repeated this 10 000 times and created a distribution of *P*-values for each factor (temperature, air pressure and their interaction). The proportion of the resulting distribution that fell below α=0.05 was the power to detect a significant effect for a given factor (Peres-Neto and Olden, 2001).

To analyze flight performance, we used an ordinal logistic regression model because our metric of flight performance was an ordered categorical response variable (flight, coerced flight, no flight). We included squared terms (appropriately centered) in the model to fit observed curvilinearity in the response variable. We compared partial deviances (χ^2 tests) of models with different combinations of main effects (temperature, air pressure, and wing loading), centered interactions, and centered squared effects to obtain the final model. All statistical analyses were done in R (2005; R Foundation for Stastical Computing, Vienna, Austria), using contributed packages Hmisc (F. E. Harrell, Jr: R package version 2.0-9, 2004), agce (R. Gottardo: R package version 1.2, 2005), MASS (Venables and Ripley, 2002), Design (F. E. Harrell, Jr: R package version 2.0-9, 2004) and multcomp (F. Bretz, T. Hothorn and P. Westfall: R package version 0.4-8, 2004).

Results

Walking performance

Among 237 female flies (range 18–20 per treatment group), walking speed ranged from 0.10 to 2.79 cm s^{-1} (at 18°C, 33%

Table 1. *ANOVA assessing the effects of temperature, air pressure and their interaction on fruit fly walking speed*

Factor (d.f.)	Summed square	*F*-value	*P*-value
Temperature (2)	15.77	73.55	<0.001
Pressure (3)	1.89	5.88	<0.001
Temperature×pressure (6)	0.77	1.2	0.307
Residuals (225)	24.13		

sea-level air pressure and 25°C, 66% sea-level air pressure, respectively). Overall, walking speed of female fruit flies increased significantly with temperature (ANOVA, $P<0.001$; Table 1, Fig. 2). On average, flies walked faster at temperatures above 18°C. However, we found no overall difference in fly walking speed between 25°C and 30°C (Tukey HSD, $P=0.15$).

Decreased air pressure significantly reduced walking speed ($P<0.001$; Table 1, Fig. 2). The depressing effect of reduced air pressure on walking speed was driven by a large decrease in walking speed at 33% sea-level air pressure (Tukey HSD, $P<0.001$; Fig. 2); no other pressures significantly reduced walking speed relative to sea-level values (Tukey HSD, all $P>0.05$). Although the overall ANOVA showed no temperature by pressure interaction effect on walking speed (Table 1), pairwise comparisons revealed interactive effects between temperature and air pressure. At 30°C flies walked significantly more slowly at 33% sea-level air pressure when compared to 66% or 100% sea-level air pressure (Tukey HSD, $P=0.012$ and $P=0.049$, respectively); however, reduced air pressure did not significantly slow walking speed at the two lower test temperatures (Tukey HSD, all $P>0.05$). This conflict between the overall ANOVA and the *post-hoc* tests may result from low statistical power. Given the size of the effect and the sample size, we had only a 48% chance of detecting a significant temperature by air pressure interaction.

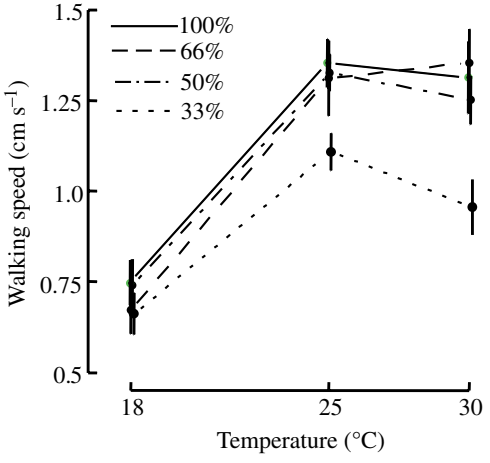

Fig. 2. Temperature and air pressure (% sea-level pressure) significantly affected walking speed of *Drosophila melanogaster* females (both $P<0.001$; Table 1). See text for measurement details. Points are shifted slightly to make vertical s.e.m. bars visible.

Flight performance

We assessed flight ability of a total of 444 flies (range of 25–30 per treatment group). All flies included in the analysis attempted at some point to fly above the fluon line (see Fig. 1). Flies were more motivated to fly at higher air temperatures and pressures (Fig. 3, open bars, 'flight'). Flies failed to fly at the lowest temperatures and at the lowest air pressures (Fig. 3, filled bars, 'no flight').

We used ordinal logistic regression to assess the effects of temperature, air pressure, wing loading and all their two-way interactions on flight performance. We also included the quadratic terms for temperature and density to account for potential curvilinearity in the response variable. We removed higher order interactions from the model if they did not significantly alter the model deviance (χ^2 test, $P<0.05$; Neter et al., 1996). The final model (Table 2) fit the flight performance data well (model likelihood ratio=233.8, 6 d.f., $P<0.001$; Nagelkerke $R^2=0.491$), accurately predicted flight

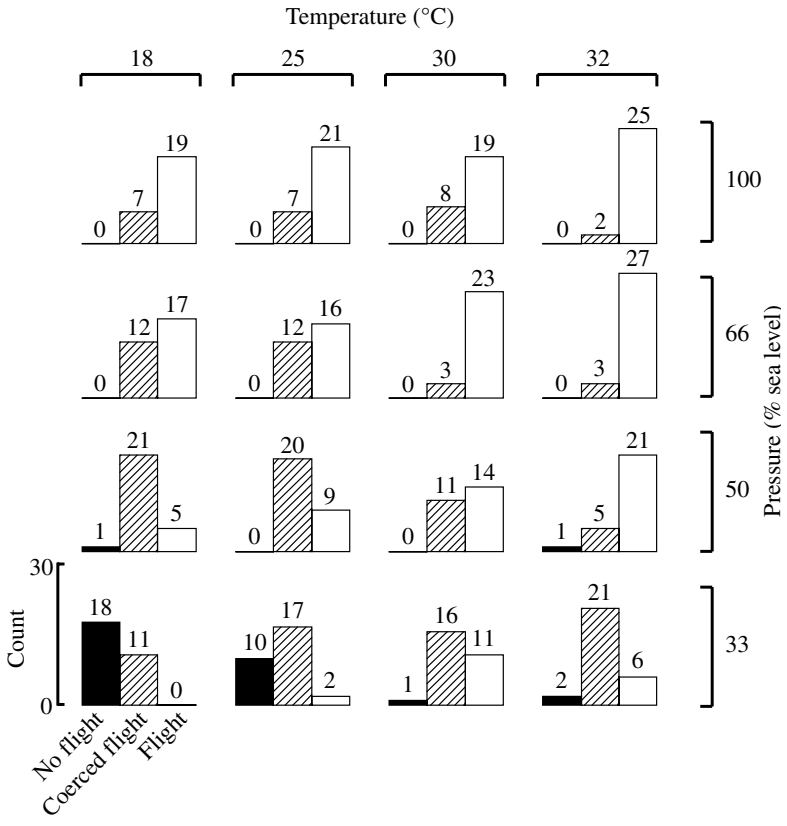

Fig. 3. Effects of temperature and air pressure (% sea-level pressure) on flight performance of *Drosophila melanogaster*. For each temperature (columns) and pressure (rows) treatment, an individual bar graph represents the counts of flies in each flight performance category: 'flight' (open bars), 'coerced flight' (hatched bars), 'no flight' (filled bars).

Table 2. *Ordinal logistic regression assessing the effects of temperature, air pressure and wing loading on fruit fly flight performance*

Factor	χ^2	d.f.	*P*-value	Odds ratio
Temperature	50.81	1	<0.001	1.219
Temperature^2	5.08	1	0.024	1.012
Air pressure	132.35	1	<0.001	1.074
Air pressure^2	43.85	1	<0.001	0.998
Temperature×pressure	12.63	1	<0.001	0.997
Wing loading	3.14	1	0.076	0.003

performance given the factors included (Goodman–Kruskal Gamma=0.693; Somers' d=0.665; τ–a=0.371), and was both sensitive (high true positive fraction) and specific (high true negative fraction, c-index=0.832; Swets, 1988).

Low temperatures reduced flight performance (Table 2, Fig. 3). Temperature also had curvilinear effects on flight performance (Temperature^2; Table 2, Fig. 3), likely due to the drop in flight performance at 32°C and 33% sea-level air pressure (Fig. 3).

Air pressure had strong linear and curvilinear effects on flight performance, with a progressively more rapid drop in flight score as pressure fell below 66% sea level (Table 2; Fig. 3).

The effect of air pressure on flight score depended strongly on air temperature. At warmer test temperatures, air pressure had little impact on flight performance; whereas at colder temperatures, reduced air pressure dramatically reduced flight performance (temperature×pressure; Table 2, Fig. 3).

We used the ordinal logistic model to predict the effects of temperature and air pressure on both the probability of flight failure ('no flight' category) and flight motivation ('flight' category). Flies were unlikely to fail except in the lowest air pressure (33% sea-level air pressure; Fig. 4). At this pressure, temperature had a strong effect on the probability of failure. At 33% sea-level air pressure and 32°C, the probability of flight failure was only 10%. At this same pressure and 18°C the probability of flight failure increased to near 60% (Fig. 4). Flight motivation (probability of flight without coercion) declined with temperature and air pressure (Fig. 5).

Flight performance was not significantly affected by wing loading despite high variation in wing loading among tested flies (*P*=0.076; Table 2, Fig. 6). However, wing loading was not distributed equally among treatments. Flies in the 32°C treatment tended to have higher wing loading than flies in the

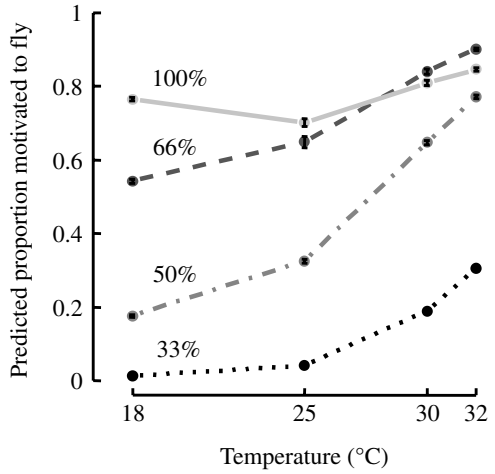

Fig. 5. Temperature and air pressure effects on flight motivation (probability of flight without coercion) for *D. melanogaster* as predicted by ordinal logistic regression. Pressures tested were 33%, 50%, 66% and 100% sea-level pressure. Values are means ± s.e.m.; see text for analysis.

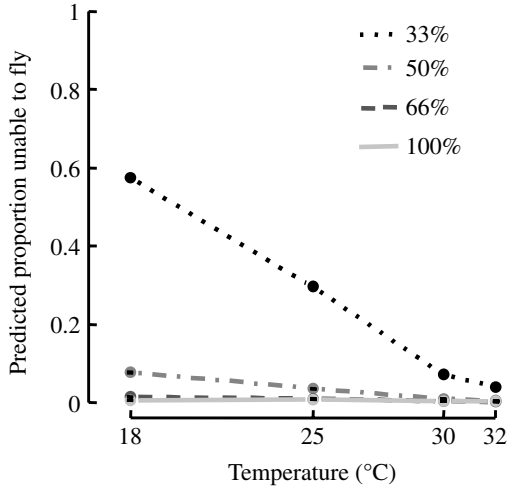

Fig. 4. Temperature and air pressure effects on flight failure (probability of 'no flight') of *D. melanogaster* as predicted by ordinal logistic regression. Pressures tested were 33%, 50%, 66% and 100% sea-level pressure. Values are means ± s.e.m.; see text for analysis.

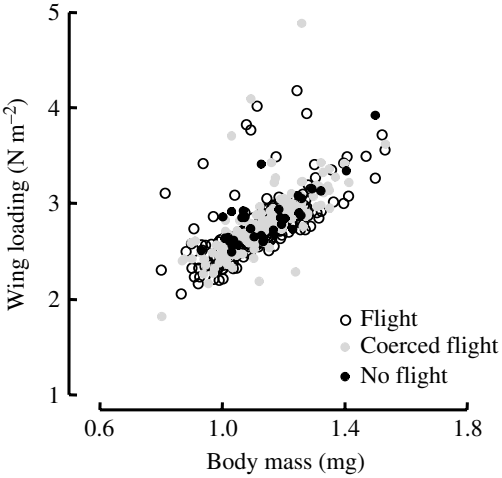

Fig. 6. Relationship between wing loading and body mass for all flies tested for flight performance. Wing loading did not affect flight performance (Table 2): 'flight' (open circles), 'coerced flight' (gray filled circles) and 'no flight' (filled circles).

18°C treatment (Tukey HSD, t=2.88, P<0.01), but there was no other significant among treatment variation in wing loading (Tukey HSD, all P>0.05). Increased wing loading should reduce flight performance. Therefore if wing loading had been evenly distributed across temperature treatments, our final conclusions would remain the same.

We additionally asked whether mass affected flight score above and beyond the effects of wing loading (e.g. Dillon and Dudley, 2004) by regressing fitted values from the final model (Table 2) on body mass. We found no significant effect of mass on flight motivation (probability of flying without coercion; ANOVA, F=0.07, P=0.80) or flight failure (probability of no flight; ANOVA, F=0.95, P=0.33), despite the large variation in body mass of flies used in the experiment (Fig. 6).

Discussion

Walking performance

Walking speed influences the ability of insects to interact socially (Partridge et al., 1987; Steele and Partridge, 1988), escape predators and obtain resources. Therefore, environmental conditions that compromise walking speed may reduce fitness. Previous work shows that colder temperatures reduce the walking speed of *D. melanogaster* (Gilchrist et al., 1997; Crill et al., 1996; Gibert et al., 2001). Low pressure may also slow walking speed because reduced P_{O_2} can affect tissue oxygen delivery. We found a positive correlation between temperature and walking speed of *D. melanogaster*, but only the lowest tested air pressure (~34 kPa; 33% sea level) significantly reduced walking speed (Table 1; Fig. 2). This is roughly equivalent to the air pressure on the peak of Mount Everest (8800 m); which fruit flies are unlikely to experience, given that populations have not been found above 5200 m (Khare et al., 2002). Therefore, walking ability at high altitudes is likely limited by temperature, but not P_{O_2}.

This result is not surprising given the empirical data that insects are generally resistant to low oxygen levels (Hoback and Stanley, 2001; Greenlee and Harrison, 2004; Klok et al., 2004). Fruit flies exposed to only 2–3% sea level P_{O_2} (1.5 kPa, less than 1/3 the oxygen level of our lowest treatment) remain active and can even fly (Farahani and Haddad, 2003). Only flies kept in anoxic conditions for more than 6 h do not fully recover (Krishnan et al., 1997). The effects of reduced P_{O_2} may be further mitigated at high altitude by increased diffusion rates at reduced air density. The reduction in P_{O_2} that occurs along an altitudinal gradient reduces the driving force for diffusion, but a concomitant drop in air density increases the diffusion coefficient, perfectly compensating (at least in theory; Denny, 1993). Insects experiencing simultaneous reductions in P_{O_2} and air density should therefore be even more tolerant to low oxygen than predicted from studies where low P_{O_2} is obtained by reducing percent oxygen while maintaining sea level air density.

We predicted that air pressure and temperature would interactively affect walking speed because insect metabolic rates increase exponentially with temperature (Berrigan and Partridge, 1997; Gillooly et al., 2001), but oxygen diffusion rates increase only slightly (Denny, 1993). Given the differential effects of temperature on these processes, we predicted that low P_{O_2} would decrease walking speed more at warmer temperatures than at cooler temperatures. This theoretical prediction is supported by developmental evidence. *D. melanogaster* reared in stressfully high temperatures are significantly larger and develop more quickly when supplied with supplemental oxygen (Frazier et al., 2001, but supplemental oxygen had little effect on these traits at lower temperatures). Similarly, *Manduca sexta* eggs appear to experience oxygen limitation at high temperatures (Woods and Hill, 2004). Our results imply that reduced P_{O_2} may also reduce walking speed at higher temperatures. Pairwise comparisons between our treatment groups revealed that flies at 30°C walked 25% more slowly at 33% than at 66% or 100% sea-level air pressure. Air pressure had no significant effect at colder temperatures. This pairwise comparison arises even though we found no significant interaction effect in the overall ANOVA (Table 1). This may reflect our low power to detect the interaction given the magnitude of the effect and the sample size.

Flight performance

Insects use flight to find mates, food and suitable oviposition sites, as well as to evade predators and to defend territories. If reduced temperatures and low air pressures compromise insect flight ability, high altitude environments would profoundly influence these fitness-related traits. We found that both low air pressure and low temperature negatively influenced the flight performance of *D. melanogaster*. In contrast to walking speed, reduced air pressure weakened flight performance at ecologically realistic levels (Fig. 3). This is likely due to aerodynamic effects of reduced air density and not due to metabolic effects of reduced P_{O_2} (Joos et al., 1997). Moreover, the combination of cold temperatures and low air pressures challenged flight more than would have been predicted by their additive effects (Table 2). At warmer temperatures, air pressure had little effect on flight performance, whereas at colder temperatures reduced air pressure caused large reductions in flight performance (Figs 3–5).

Except in the most extreme conditions, most flies (>80%) successfully initiated flight with or without agitation (Fig. 4). Flies failed regularly only in 33% sea level pressure when temperature was at or below 25°C. Remarkably, even at 50% sea-level air pressure and 18°C, 90% of *D. melanogaster* could fly, even though this reduction in air density requires about a twofold increase in lift production. The limited effects of low temperature and low pressure on flight failure may reflect the conservative nature of our assay. Flies did not have to sustain a long flight to arrive above the fluon line (Fig. 1).

The ability of *D. melanogaster* to fly in low temperature and low pressure also reinforces the general finding that insects have a remarkable capacity for augmenting flight performance (Lehmann, 1999; Dudley, 1995; Dillon and Dudley, 2004). Insects can augment force production by increasing stroke

amplitude (Dudley, 1995; Dillon and Dudley, 2004). In heliox (20.9% oxygen, balance helium), which is approximately equivalent in density to our 33% treatment, hovering orchid bees increased stroke amplitude by as much as 31% (Dudley, 1995). Similarly, flies produce maximum forces in response to loading by increasing stroke amplitude (Lehmann, 1999). Alternatively, insects can increase wing-beat frequency, but for ectotherms this may be impossible in cold conditions (Curtsinger and Laurie-Ahlberg, 1981). At 15°C half of *D. melanogaster* tested could not maintain flight for 1 s, likely due to reduced wing-beat frequencies (Lehmann, 1999; Curtsinger and Laurie-Ahlberg, 1981). More subtle changes in wing-beat kinematics may also allow flies to increase force production to fly in high altitude conditions (Sane and Dickinson, 2001). However, all of these kinematic adjustments are likely to be energetically costly (Dudley, 1995; Chadwick and Williams, 1949; Chadwick, 1951).

Compared to flight failure, flight motivation was highly sensitive to temperature and pressure (Figs 3, 5). As temperature and air pressure declined, flies became increasingly unwilling to fly without agitation (Fig. 5). The combination of cold temperatures and low air pressures reduced flight motivation more than predicted by the additive effects of these factors (Fig. 5). This large interactive effect may reflect the combination of two challenges. To fly in reduced air pressure, insects must produce greater muscle forces to alter wing-beat kinematics (Dudley, 2000), but their ability to do so is hampered by reduced physiological reaction rates at cold temperatures (e.g. Huey and Kingsolver, 1989; Berrigan and Partridge, 1997; Josephson, 1981; Hosler et al., 2000). Flies are physiologically compromised at the same time that demand for performance is high, reducing their motivation to fly.

The need to conserve water may also reduce flight motivation in high altitude conditions. Reduced and pressure at altitude both increase the driving force for evaporative water loss (for a review of water balance issues in insects, see Sømme, 1995). Insects minimize water loss by keeping their spiracles closed except during brief periods of gas exchange (Lighton, 1996). However, increased metabolic demand from the flight muscles and reduced atmospheric P_{O_2} may drive insects to increase the frequency and duration of spiracular opening, increasing water loss (Joos et al., 1997). For example, *Drosophila* species lose water more than 3.5 times faster when hovering than when at rest and an additional 20% faster during elevated force production (Lehmann et al., 2000). These effects may be more pronounced at high altitude where force requirements for flight are likely higher, and where the driving force for evaporative water loss is greatly increased.

Insects may compensate for challenging flight conditions at high altitude by reducing wing loading (Dudley, 2000; insect weight/wing area). Theoretically, reduced wing loading will reduce induced power requirements while also allowing for increased lift production (for a review, see Dudley, 2000). For this reason, well-documented geographic and developmental variation in wing loading of *Drosophila* has been hypothesized to be adaptive (Norry et al., 2001; Loeschcke et al., 1999; Starmer and Wolf, 1989). However, wing loading did not affect maximum take-off performance of 70 species of birds, bats and insects (Marden, 1987) or maximum load-lifting performance of 11 bee species (Dillon and Dudley, 2004). Naturally occurring variation in wing loading in our population of fruit flies (Fig. 6) allowed us to explicitly test the effect of wing loading on flight performance. We found no significant effect of wing loading on flight performance (Table 2), despite significant variation in our population.

Implications for life at high altitude

High altitude environments have long been equated to high latitude environments because of their climatic similarities (Hopkins, 1938), but these two environments may have very different physiological effects on insects. Our results suggest that high altitudes are likely more challenging than high latitudes, at least for *D. melanogaster*. The independent and interactive effects of low temperature and low air pressure dramatically reduced flight motivation, and increased the probability of flight failure. This interaction may lead to different predictions for insect thermoregulation at high altitudes *vs* high latitudes at sea level. For instance, small flying insects may need to maintain comparatively warmer body temperatures at high altitudes, due to the profound effects of low air pressure when combined with low temperatures on flight performance.

The interactive effects of temperature and air pressure on insect flight performance may help explain the increased prevalence of flightless insects at high altitude. Historically, the evolution of flightlessness at high altitudes has mostly been attributed to prevailing environmental conditions such as increased wind, cold temperatures and low air pressures (reviewed by Mani, 1968; Sømme, 1989). These conditions are generally acknowledged to make flight more difficult, or impossible; and wind may also be risky for flying insects, causing unintended transport to unfavorable locations. Our results confirm that cold temperatures and low air pressures – and especially the combination of the two – do indeed challenge insect flight. But our findings also suggest that ‘behavioral drive’ may provide an explicit explanation for why these challenging environmental conditions promote the evolution of wingless and flightless insects at high altitude. This classical theory posits that changes in behavior drive evolutionary change in other traits (Mayr, 1963). High altitude conditions dramatically reduced flight motivation of *D. melanogaster* (Fig. 5). If insects generally avoid flying at high altitudes they will not enjoy the benefits of flight (finding food and mates, escaping predators), but they will continue to incur the costs of developing and maintaining the flight machinery. Selection should then favor reduction or loss of the unused flight machinery, increasing the probability of evolving flightlessness over evolutionary time.

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