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Cold rearing improves cold-flight performance in *Drosophila via* changes in wing morphology

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SUMMARY

We use a factorial experimental design to test whether rearing at colder temperatures shifts the lower thermal envelope for flight of *Drosophila melanogaster* Meigen to colder temperatures. *D. melanogaster* that developed in colder temperatures (15°C) had a significant flight advantage in cold air compared to flies that developed in warmer temperatures (28°C). At 14°C, cold-reared flies failed to perform a take-off flight ~47% of the time whereas warm-reared flies failed ~94% of the time. At 18°C, cold- and warm-reared flies performed equally well. We also compared several traits in cold- and warm-developing flies to determine if cold-developing flies had better flight performance at cold temperatures due to changes in body mass, wing length, wing loading, relative flight muscle mass or wing-beat frequency. The improved ability to fly at low temperatures was associated with a dramatic increase in wing area and an increase in wing length (after controlling for wing area). Flies that developed at 15°C had ~25% more wing area than similarly sized flies that developed at 28°C. Cold-reared flies had slower wing-beat frequencies than similarly sized flies from warmer developmental environments, whereas other traits did not vary with developmental temperature. These results demonstrate that developmental plasticity in wing dimensions contributes to the improved flight performance of *D. melanogaster* at cold temperatures, and ultimately, may help *D. melanogaster* live in a wide range of thermal environments.

Key words: beneficial acclimation, developmental plasticity, wing loading, wing-beat frequency, body size, free flight, temperature.

INTRODUCTION

Insects rely on flight to find mates, interact socially, evade predators and obtain resources; when flight performance is compromised these fitness-related traits will be impaired. Cold temperatures challenge the flight performance of ectothermic insects, yet many volant species inhabit cold environments. To fly in such environments insects must compensate with adjustments in morphology and physiology *via* the processes of local genetic adaptation, acclimatization and developmental plasticity (Harrison and Roberts, 2000). In this study, we tested whether developmental plasticity – the physiological and morphological responses of a single genotype to an organism's environment during development (Stearns, 1992) – can extend the thermal performance envelope of insects that must fly in the cold. Specifically, we tested whether *Drosophila melanogaster* Meigen that develop at colder temperatures have better free-flight performance in cold air compared to those that develop at warmer temperatures.

The beneficial plasticity hypothesis suggests that developmental plasticity should give organisms a competitive advantage in the environment in which they develop (Leroi et al., 1994). However, many experiments that have explicitly tested for beneficial plasticity or acclimation have rejected this hypothesis (Blanckenhorn, 2000; Gibbs et al., 1998; Gibert et al., 2001; Huey et al., 1995; Leroi et al., 1994; Woods, 1999; Woods and Harrison, 2001; Zamudio et al., 1995) or have had mixed results (Bennett and Lenski, 1997; Carter and Wilson, 2006; Deere and Chown, 2006; Deere et al., 2006; Stillwell and Fox, 2005). Together these studies suggest that alternative hypotheses, such as "colder/hotter is better," or "optimal developmental temperature" may be evolutionarily more important than beneficial plasticity and acclimation (Huey et al., 1999). This intuitive hypothesis may lack experimental support for a number of reasons. These include potentially high costs of plasticity, unreliable or insensitive cues triggering plasticity, evolutionary constraints, and long-term negative effects of non-optimal conditions on organisms (DeWitt et al., 1998; Wilson and Franklin, 2002; Woods and Harrison, 2002).

Another possible explanation for the rejection of the beneficial acclimation hypothesis in many experimental tests is that the benefits of developmental plasticity may occur at temperatures more extreme than the developmental temperatures. Ectotherms experience daily and seasonally varying temperatures, and successful ecological performance will depend partly on the breadth of their thermal performance range. Stochastic weather events are major factors determining population sizes of many insects (Price, 1997), suggesting that the ability to survive extremes may be very important components of fitness in nature for ectotherms such as insects. We hypothesized that developmental plasticity provides a benefit by extending the thermal range of the insect in the direction of stress. For adult fruit flies, flight is critical for feeding and mating, and therefore fitness. In this study, we test whether rearing sub-adult fruit flies at colder temperatures correspondingly shifts the lower limits of adult flight performance to colder temperatures.

Flying at cold temperatures is challenging for ectothermic insects because cold temperatures impair the contractile properties of

muscle (Josephson, 1981), resulting in lower wing-beat frequencies and reduced power output during tethered (Curtsinger and Laurie-Ahlberg, 1981) and free flight (Lehmann, 1999). As temperatures decline, fruit flies become less motivated to initiate flight and more likely to experience flight failure (Dillon and Frazier, 2006). Potentially compounding the problem, most ectothermic species mature at larger body sizes when they develop in cold temperatures (Atkinson, 1994), and therefore must generate more power to support their extra body weight during flight (Dillon and Dudley, 2004).

Developmental plasticity could potentially help insects compensate for the challenges of flying at cold temperatures in several ways. Flight muscle performance could improve at cold temperatures through changes in biochemistry (Hochachka and Somero, 2002; Laurie-Ahlberg et al., 1985; Rogers et al., 2004), or the mass of flight muscle relative to body mass may increase (Marden, 1987). These changes could translate into increased force and power production through changes in gross kinematics (wing-beat frequency and stroke amplitude) or more subtle changes in the three dimensional motions of the wings (Dickinson et al., 1999; Dudley, 2000; Sane, 2003). Insects from cold environments could also increase wing area relative to body mass (i.e. decreased wing loading, body weight per wing area; N m^{-2}), which should reduce induced power requirements and increase lift production (Dudley, 2000). Indeed, insects that develop in cold temperatures tend to have lower wing loading as a result of both evolutionary and plastic responses (Azevedo et al., 1998; David et al., 1994; Gilchrist and Huey, 2004; Loeschcke et al., 1999; Morin et al., 1999; Norry et al., 2001; Petavy et al., 1997; Stalker, 1980; Starmer and Wolf, 1989). Changes in wing shape may also improve flight performance. For example, elongating the wing, while maintaining the same wing area, should theoretically improve some aspects of flight performance because the higher translational velocity of the wing tips (at the same angular velocity) yields greater aerodynamic forces (Ellington, 1984; Pennycuick, 1968).

To test our main prediction that cold-rearing improves flight performance at lower temperatures, we reared *D. melanogaster* at three ecologically realistic temperatures (15, 23 and 28°C) and then tested whether they could initiate a free-flight across a range of cold temperatures around 16°C, reported to be the minimal temperature at which flies could generate sufficient lift for free flight (Lehmann, 1999). We chose developmental temperatures that are commonly experienced by this species in the field and lab, and that do not reduce egg to adult survival (Frazier et al., 2001) to avoid possible stressful effects (Wilson and Franklin, 2002). Furthermore, we examined body, wing and muscle morphology, and wing-beat frequency during flight, to determine how thermally dependent variation in these traits may contribute to flight success in cold temperatures.

MATERIALS AND METHODS

Experimental animals

The *Drosophila melanogaster* used in this experiment were collected as eggs from an Ives strain obtained from TA Markow [see Coyne et al. (Coyne et al., 1993) for information about strain]. Flies were maintained in the laboratory at room temperature [~24°C; see Frazier et al. (Frazier et al., 2001) for colony and egg collection information]. The eggs were placed 20 per vial (9.5 cm long, 2.2 cm diameter, with ~9 ml dextrose diet) to control population density. These flies were then reared at 15, 23 or 28°C (T_{dev}) in temperature-controlled incubators under a 14 h:10 h L:D photoperiod. Survival rates are not affected by these developmental temperatures (Frazier et al., 2001). We collected the same number of eggs for each developmental temperature. As flies began emerging, we transferred the adults to fresh food vials every 8 h to control for age. For the flight assay, we used the flies in the order that they emerged, including the earliest emerging flies from all treatment groups [these first flies tend to be smaller (Chippendale et al., 1997)]. We reared more adult flies than we tested and consequently the slowest developing flies from each temperature treatment were excluded from the study.

Males and females were separated to prevent mating. We allowed the flies to mature for 48–72 h before starting flight assays, because wing-beat frequency and power output increases until 2 days of age and then remains constant from 2 to 8 days of age (Curtsinger and Laurie-Ahlberg, 1981). During the adult maturation period, all flies were held at 22°C to ensure that any developmental effects were due to beneficial plasticity rather than reversible, short-term acclimation after emergence [“phenotypic flexibility” (Piersma and Drent, 2003)].

Flight assay

To evaluate flight performance at cold temperatures, flies were randomly assigned to one of three flight test temperatures: 14, 16 or 18°C (T_{test}; respective means ± s.d.: 14.5±0.29, 16.02±0.22, 18.16±0.20; based on the mean start and end temperature recording for each flight test). Individual flies were aspirated into a covered 500 ml water-jacketed beaker (Konte, Vineland, New Jersey, USA), the jacket of which was continually flushed with temperature controlled water. This flight chamber was housed in a temperature-controlled incubator. The temperature inside the flight chamber was monitored throughout the experiment using a calibrated thermocouple thermometer (Physitemp Bat-12, Bailey Instruments Inc., Saddlebrook, NJ, USA) to ensure that the temperature did not significantly deviate from the T_{test}. The water-jacketed beaker successfully buffered the temperature inside the flight arena, across all treatments, the differences between the highest and lowest temperature during a flight test averaged 0.40±0.33°C (mean ± s.d.). After a 1 min thermal equilibration period, we encouraged escape behavior by chasing the fly with the tip of a fine, thermally equilibrated paintbrush inserted into the beaker through an opening in a piece of rubber covering the top of the beaker. We scored the flight performance of each fly, placing it in one of three categories: those that could fly the full width of the beaker (8 cm) were categorized as performing a ‘flight’; those that flew >5 cm but <8 cm, stereotypically a take-off followed by an arching loop ending on the chamber floor, were categorized as generating ‘lift’ (these flies were unable to sustain flight, but we considered this behavior distinct from ‘failed’ flight because they traveled further than the maximum jumping distance observed in preliminary experiments with wingless flies); flies that traveled <5 cm were ‘failed’ fliers because they were unable to generate any lift and could do little more than jump off of the bottom of the chamber. We continued chasing the fly until it performed a flight or 5 min had passed.

Morphological and physiological data

We immediately weighed each fly after the flight assay, on a Cahn C-33 microbalance (±2 μg; Cahn Instruments, Inc., Cerritos, CA, USA) and then preserved the fly in 70% ethanol. For measures of wing morphology, both wings were removed and mounted on slides. Total wing area and wing length for each fly was quantified to the nearest 2 μm using a computer-controlled microscope-mounted digital camera and Scion Image software (Scion Corporation, Frederick, MD, USA). We estimated flight muscle ratio (FMR) as the ratio of dry thorax mass to dry body mass. The head, thorax and abdomen were separated and dried for 24 h at 55°C, then immediately weighed using the Cahn microbalance. The thorax primarily houses flight muscle and thus provides an index of flight muscle mass.

We measured the wing-beat frequencies (WBF) of the flies that performed a flight or generated lift with an optical tachometer, which converted fluctuations in light due to wing beats into a sound recording on tape (Unwin and Ellington, 1979). A battery-powered light was wrapped with a white piece of paper and positioned directly behind the flight chamber. This provided diffuse lighting and a high-contrast background that was optimal for operating the tachometer. The optical tachometer recordings were digitized and visualized using the SpectraPLUS sound analysis program (Pioneer Hill Software, Poulsbo, Washington DC, USA) as previously described (Roberts, 2005; Roberts et al., 1998; Roberts et al., 2004). Each recorded sequence contained 6–10 clearly distinguishable, uninterrupted wing beats. For a given fly, WBF was determined to the nearest 0.2 Hz by dividing the number of clearly distinguishable, uninterrupted wing beats in the sequence by the duration of the sequence (measured to the nearest 0.0001 s).

Data analysis

To analyze the effects of flight temperature (T_{test}) and developmental temperature (T_{dev}) on flight performance, we used an ordinal logistic regression model because our metric of flight performance was an ordered categorical response variable. We included in the model appropriately centered interaction terms to test for beneficial acclimation ($T_{test}\times T_{dev}$) and squared terms to fit observed curvilinearity in the response variable. Statistical analyses were done in R (R: A Language and Environment for Statistical Computing, 2005; version 2.1.0; R Foundation for Statistical Computing, Vienna, Austria), using contributed packages Hmisc (Harrell Miscellaneous; F. E. Harrell, Jr: R package version 3.0–7, 2005); agce (Analysis of Growth Curve Experiments; R. Gottardo: R package version 1.2); MASS [Modern Applied Statistics with S (Venables and Ripley, 2002)]; and Design (Design Package; F. E. Harrell, Jr: R package version 2.0-12, 2005). We used ordinary least squares regression to analyze morphological and physiological variation of traits in response to T_{test}, T_{dev}, gender and body size. For all analyses, we compared partial deviances (χ^2 tests) of models with different combinations of main effects, interactions and squared effects to obtain the final model. Type I error was set at 0.05.

RESULTS

Flight performance

Flies developed in one of three temperatures (T_{dev}: 15, 23, 28°C), and we categorized the flight performance of each fly at a single air temperature (T_{test}: 14, 16, 18°C), for a total of nine treatment groups. There were 30–35 flies in each treatment group and a total of 282 flies (Fig. 1).

We used ordinal logistic regression to assess the effects of test temperature, development temperature and gender on flight performance (Table 1). The final model fitted the flight performance data well (model likelihood ratio χ^2=243.7; d.f.=6, 282; Nagelkerke R^2=0.658), and accurately predicted flight performance based on measures of association (Goodman–Kruskal gamma=0.82; Somers' D=0.796; Kendall's τ-a rank correlations between predicted probabilities and observed responses=0.509), and the average sensitivity over all possible specificities was high [c-index=0.898, i.e. area under ROC curve (Swets, 1988)].

Test temperature had the largest effect on *D. melanogaster* flight performance (Fig. 1, Table 1; T_{test}, P<0.0001). At the lowest test temperature (14°C), only ~3% of the flies were able to fly, whereas, at the highest test temperature (18°C) nearly all flies were able to fly (~93%). Developmental temperature also influenced the flight performance of *D. melanogaster*. Flies that developed at 15°C had the highest probability of flying at the coldest test temperature, indicating beneficial plasticity (Fig. 1, Table 1; $T_{test}\times T_{dev}$, P=0.0019). At 14°C, cold-reared (15°C) flies failed ~47% of the time, whereas warm-reared (28°C) flies failed ~94% of the time. A similar trend was observed at 16°C; flies reared at 15°C failed ~6% of the time, whereas flies reared at 28°C failed ~33% of the time. Development temperature also had nonlinear effects (squared terms) on flight performance; flight performance decreased more as developmental temperature went from 28 to 23°C than from 23 to 15°C (Fig. 1, Table 1; T_{dev}^2, P=0.0041).

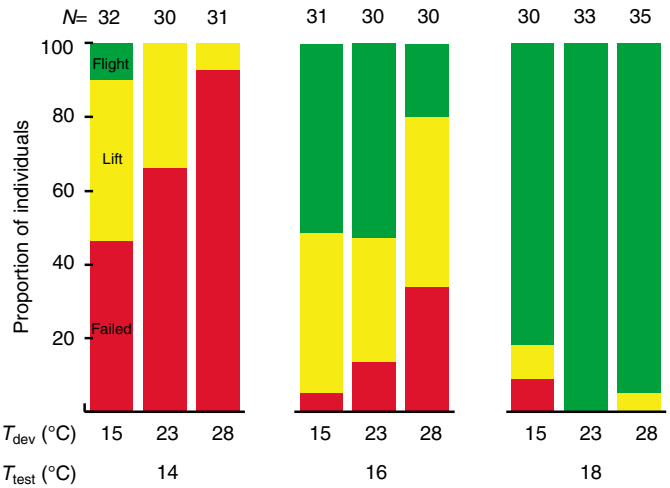

Fig. 1. Effects of test temperature (T_{test}) and development temperature (T_{dev}) on flight performance of *D. melanogaster*. Green indicates the proportion of flies that were able to perform a flight, yellow indicates lift generation (but not flight), red indicates flight failure (see Materials and methods for details). Flies that developed in colder temperatures had significantly better flight performance in colder temperatures (see Table 1), indicating beneficial plasticity.

Temperature effects on morphology and WBF

D. melanogaster were larger when they developed in cold temperatures (Fig. 2, Table 2A; T_{dev}, P<0.001). Females were also significantly larger than males (Fig. 2, Table 2A; gender, P<0.001), and there was a significant interaction between gender and development temperature on body mass (Table 2A; $T_{dev}\times$gender, P<0.001), suggesting that developmental temperature affected males and females differently. Specifically, male body size increased relatively more than did female body size at 15°C *versus* 23°C developmental temperatures.

Wing area was larger in cold-reared flies (Fig. 2A), and more than compensated for the minor increase in body size. As a result, flies developing at cold temperatures had the lowest wing loading (Fig. 2B, Table 2B; T_{dev}). This result contrasts with the relationship of the wing loading to body mass within a developmental temperature, which is a positive relationship (Fig. 2B, red, orange and blue lines; Table 2B, ln mass). This suggests that larger flies, when development occurs at the same temperature, may be at a disadvantage when it comes to flight. For their size, males had relatively higher wing loading than females. At a rearing temperature of 23°C, males are predicted to have about 8% greater wing loading than a similarly sized female. One potentially confounding issue is that body mass varies with food consumption (David et al., 2006), which was not controlled in our study. To test the possibility that the increased wing loading in larger flies was due to transient increases in weight, we substituted thorax mass (which should not vary with prior meal size) for body mass in a regression model (including gender and rearing temperature). There

was a positive correlation between thorax mass and wing loading (P=0.011), further supporting the finding that larger flies (within a developmental temperature) have higher wing loading and perhaps are disadvantaged at flight.

Similarly, the scaling relationship between body mass and wing area depended on whether variation in these two variables was due to development temperature or to other factors. When the variation in body size and wing area was due to developmental temperature, wing area scaled with mass$^{2.41}$ (based on a regression analysis using the mean body mass and mean wing area at each developmental temperature and for each gender, i.e. the open black circles on Fig. 2A; 95% confidence interval: 1.33–3.49, N=6). This scaling relationship favors the flight performance of the larger, cold-reared flies. Relative to body mass, the wings of the cold-reared flies were much larger than predicted on the basis of the theoretical scaling relationships and the scaling relationship we observed between wing area and body mass within a rearing temperature. Based on our data, within each developmental temperature, wing area scaled with mass$^{0.32}$ (calculated from the mean of the slopes from each T_{dev} and gender group, i.e. the solid red, orange and blue lines in Fig. 2A, N=6).

Another potential mechanism insects may use to improve flight performance in cold temperatures is increasing the proportional length of their wings. According to a regression analysis (Table 2C), flies from colder developmental temperatures had relatively longer wings (P<0.001), even after statistically controlling for the increase in overall wing area (not surprisingly, wing length was strongly correlated with wing area, Pearson's correlation coefficient=0.94, N=228). Males had relatively shorter wings than females (P<0.001).

Flying insects could also increase their flight muscle ratio (FMR, mass thorax/mass body) to generate more power for flight. We predicted that flies from cold developmental temperatures would have a higher FMR. On average, thorax dry mass was ~50% (±8.3% s.d., N=282) of total body dry mass and this ratio was not correlated with developmental temperature (P=0.379), mass (P=0.222) or gender (P=0.343).

Although the percentage of thorax (and presumably flight muscle) remains constant, insects developing in cold environments might have increased WBFs due to changes in muscle physiology. For this analysis, we included flies that performed a 'flight' and those that generated 'lift' (those that failed to fly were excluded); we combined these two groups in the analysis because they did not have significantly different WBFs (P=0.88). WBF declined with decreasing flight temperature (Fig. 3, Table 2D; T_{test}, P<0.001), as observed in other studies (Curtsinger and Laurie-Ahlberg, 1981; Laurie-Ahlberg et al., 1985; Lehmann, 1999; Stevenson and Josephson, 1990; Unwin and Corbet, 1984). Flies that developed at 15°C, had significantly lower WBFs than flies that developed at the warmer temperatures (Fig. 3, blue *vs* orange and red points; Table 2D; T_{dev}, P=0.020); however, there was no significant difference between flies that developed at 23 *vs* 28°C. Interestingly, larger flies had faster WBFs, whereas flies with larger wing areas had slower WBFs. These data demonstrate that cold-reared fruit flies do not compensate for colder flight temperatures by increasing wing-beat frequency.

Table 1. Ordinal logistic regression assessing the effects of gender, test temperature, developmental temperature and their interaction (i.e. beneficial plasticity) on fruit fly flight performance

Variable (d.f.)	Coefficient	s.e.m.	χ^2	P-value	Odds ratio
T_{test} (1)	1.527	0.181	109.24	<0.0001	4.605
T_{dev} (1)	–0.121	0.038	12.48	0.0016	0.886
$T_{test}\times T_{dev}$ (1)	0.081	0.026	13.43	0.0019	1.084
T_{test}^2 (1)	0.013	0.093	0.03	0.8875	1.013
T_{dev}^2 (1)	–0.027	0.009	9.40	0.0041	0.974
Gender (1)	–0.242	0.309	0.70	0.4337	1.274

T_{test}, test temperature; T_{dev}, developmental temperature. Estimates of standard error (s.e.m.) and P-value are bootstrapped.

DISCUSSION

D. melanogaster reared at colder temperatures were able to initiate flight at temperatures that were prohibitively cold for flies reared at warmer temperatures (Fig. 1, Table 1). Thus, a beneficial aspect of this developmental plasticity is an expanded thermal performance window. At 18°C test temperatures, the flight performance of warm- and cold-reared flies did not differ (nearly all flies could fly). However, at 16°C, 52% of cold-reared flies could initiate flight,

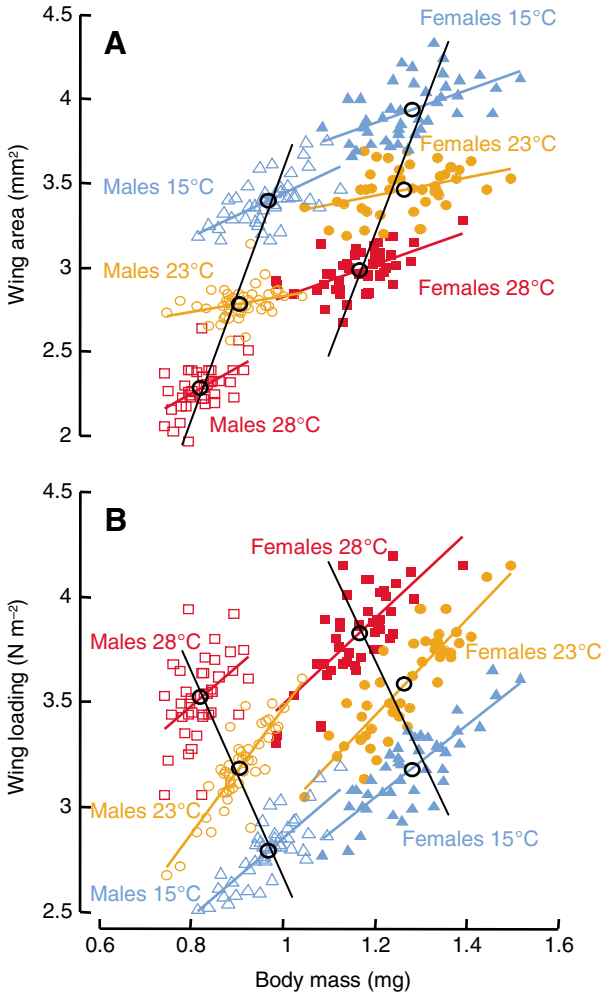

Fig. 2. Effects of body mass, development temperature and gender (females, filled symbols; males, open symbols) on (A) wing area and (B) wing loading of *D. melanogaster*. When wing loading was compared within a single developmental temperature (T_{dev}: 15°C, blue triangles; 23°C, orange circles, 28°C, red squares) larger flies had greater wing loading. However, this scaling relationship was dramatically altered when variation in wing area and body size was due to development temperature. Flies from colder temperatures had much lower wing loading (black lines indicate relationships across developmental temperatures). The black circles indicate mean body mass and wing area/loading for each treatment group.

Table 2. Regression models for factors affecting: (A) mass, (B) wing loading, (C) wing length and (D) wing beat frequency in *D. melanogaster*

	Variable*	Parameter estimate	s.e.m.	*t*-value	*P*-value
A. ln mass (mg):					
$F_{4,277}$=438, P<0.001, R^2=0.86	Intercept	0.4172	0.02778	15.02	<0.001
	T_{dev} (°C)	–0.0082	0.00112	–7.31	<0.001
	T_{dev}^2	–0.0010	0.00022	–4.58	<0.001
	Gender (males)	–0.1959	0.03441	–5.69	<0.001
	T_{dev} × gender	–0.0057	0.00152	–3.76	<0.001
B. Wing loading (N m^{-2}):					
$F_{3,254}$=530, P<0.001, R^2=0.86	Intercept	1.5645	0.06646	23.54	<0.001
	T_{dev} (°C)	0.0707	0.00210	33.70	<0.001
	ln mass (mg)	1.9622	0.12755	15.38	<0.001
	Gender (males)	0.2623	0.04481	5.86	<0.001
C. Wing length (mm):					
$F_{3,225}$=680, P<0.001, R^2=0.90	Intercept	1.7918	0.15218	11.77	<0.001
	T_{dev} (°C)	–0.0088	0.00241	–3.64	<0.001
	Wing area (mm^2)	0.2326	0.02918	7.97	<0.001
	Gender (males)	–0.1051	0.02052	–5.12	<0.001
D. Wing beat frequency† (s^{-1}):					
$F_{5,102}$=86.23, P<0.001, R^2=0.81	Intercept	142.4066	18.3380	7.77	<0.001
	T_{dev} (°C) 15°C	–8.1627	3.4674	–2.35	0.020
	T_{test} (°C)	7.7525	0.5902	13.14	<0.001
	ln mass (mg)	55.9370	13.9107	4.02	<0.001
	Wing area (mm^2)	–24.5410	4.8130	–5.10	<0.001
	Gender	3.6741	3.7259	0.99	0.326

F, P, and R^2 regression statistics are provided for the overall model, followed by the parameter estimates, standard error, *t*-value, and *P*-value for each variable within the model.

*If parameter estimates are used for prediction purposes, the following should be noted: variables with squared terms are centered on the variable's mean, consequently, T_{dev}^2 equals $(T_{dev}–22.1)^2$. When estimates are calculated for males the parameter estimate for gender is included in the model, for females the parameter estimate becomes 0. Because the regression estimates for T_{dev} are based on only three temperatures, caution should be exercised if these models are used for prediction at developmental temperatures other than 15, 23, or 28°C.

†Includes flies from both the 'flight' and 'lift' categories of performance, but not 'failed' flight. Flies that performed a 'flight' or generated 'lift' are grouped in the analysis because there was no significant difference in wing-beat frequency (WBF) between these flies (when flight performance was included as a variable in the above analysis, P=0.88). Flies that developed at 23°C and 28°C were grouped because WBF differed by only 1.3 beats per second as estimated by the model and was not significantly different.

whereas only 20% of the warm-reared flies could. The difference became more dramatic as test temperature decreased: at 14°C, more than 50% of the cold-reared animals could fly or generate lift, while ~94% of warm-reared flies could not.

Decreased wing loading (and increased wing area) in *Drosophila* spp. in response to cold developmental temperatures has been observed in multiple studies (Barnes and Laurie-Ahlberg, 1986; David et al., 1994; Gilchrist and Huey, 2004; Petavy, 1997). Owing to the theoretical advantages of reduced wing loading for generating lift during flight (Dudley, 2000) and increasing mechanical power output (Barnes and Laurie-Ahlberg, 1986), this response has been hypothesized to be adaptive for flight (Loeschcke et al., 1999; Norry et al., 2001; Starmer and Wolf, 1989). However, reduced wing loading is not always associated with improved flight performance (Dillon and Dudley, 2004; Dillon and Frazier, 2006; Marden, 1987). To our knowledge, this is the first study to experimentally demonstrate that the increased wing dimensions that occur with cold-rearing in flies results in improved flight performance, specifically by making flight possible at lower temperatures.

Within a single developmental temperature, larger flies had greater wing loading (Fig. 2); however, at colder developmental temperatures flies were larger but had much lower wing loading due to a dramatic increase in wing area. This clearly shows differential regulation of wing *vs* body morphological development in response to temperature (see also David et al., 2006). In order to aerodynamically compensate for the increase in body size (assuming all else remains equal) the wing area of flies from cold environments must scale isometrically with body mass (wing area $\propto$ mass^1) because lift is directly proportional to wing area (Denny, 1993; Dudley, 2000). In fact, when variation in wing area and body mass was due to developmental temperature, wing area scaled with mass$^{2.41}$ (black lines in Fig. 2A; values in figure are not logged, however, we used the natural log of the values to determine scaling relationships). This scaling relationship is surprising given the expectation that the scaling coefficient should be ~0.66 based on dimensional analysis predictions (Pennycuick, 1992) and <1 based on empirical data from comparative studies both within and among species (Casey and Joos, 1983; Dillon and Dudley, 2004; Dillon and Frazier, 2006; Dudley, 2000; Gilchrist and Huey, 2004; Starmer and Wolf, 1989). The greater wing area would be advantageous in the cold because flies can generate increased lift despite the decreased muscle power output that occurs at colder temperatures (Lehmann, 1999).

The shape of the wings also appears to change in response to developmental temperature such that flies that develop at cold temperatures have longer wings, even after controlling for the increase in wing area (Table 2). This is consistent with their improved flight performance at cold temperatures because wing tips have higher translational velocity (at the same angular velocity) and yield greater aerodynamic forces (Ellington, 1984; Pennycuick, 1968). According to model predictions, flies that developed at 15°C have about 8% longer wings than flies at 28°C, even when wing area is controlled for.

Flies that developed in cold temperatures had significantly lower wing-beat frequencies than flies that developed at warm temperatures at all test temperatures (Fig. 3, Table 2C). This result is consistent with Barnes and Laurie-Ahlberg's study (Barnes and Laurie-Ahlberg, 1986), and may be explained by the fact that the flies that developed in cold temperatures were heavier and had larger wings; and larger insects tend to have reduced wing-beat frequencies (Dillon and Dudley, 2004; Dudley, 2000; Petavy et al., 1997) because of resonance issues and an increase in the induced power required to move a larger wing. Indeed, within a developmental temperature, flies with larger wings had slower wing-beat frequencies (Table 2D, wing area; Fig. 3B), however, heavier flies appeared to have faster wing-beat frequencies (Table 2D, ln mass; Fig. 3A), perhaps due to their higher wing loading. Similarly, heavier carpenter bees have higher wing loading and wing-beat frequencies during hovering (Roberts et al., 2004).

D. melanogaster that develop in cold environments could still have physiological mechanisms that improve flight muscle

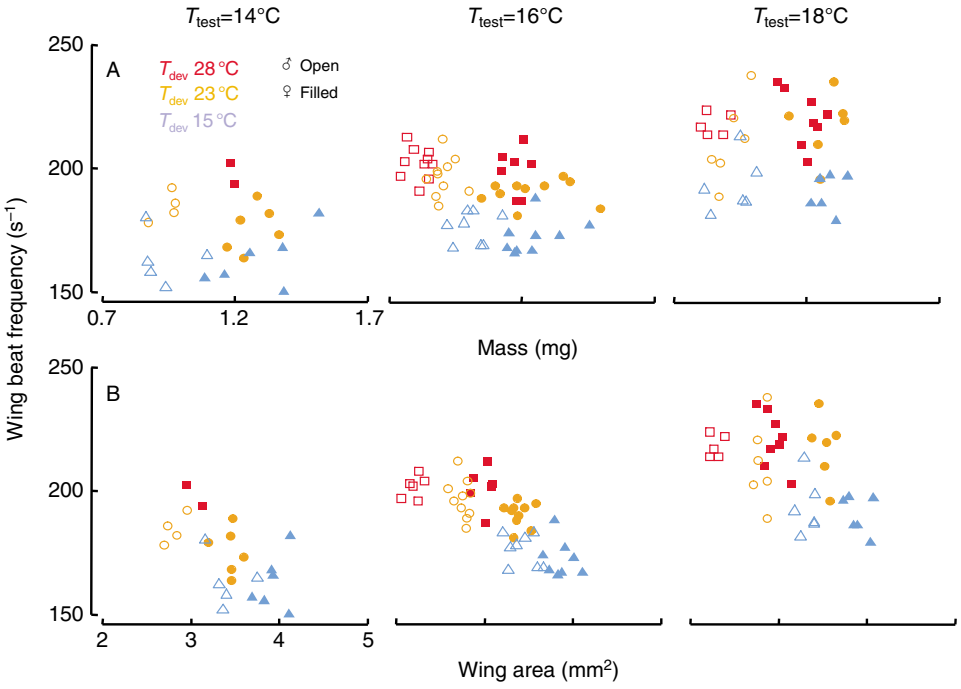

Fig. 3. Wing-beat frequency (WBF; s^{-1}) of *D. melanogaster* as a function of flight temperature (T_{test}, °C), developmental temperature (T_{dev}, °C), body mass (A) and wing area (B). As test temperature increased, wing-beat frequency significantly increased (plots from left to right). Flies developing at cold temperatures (blue triangles, 15°C) had significantly lower wing-beat frequencies at every test temperature compared with flies developing at intermediate temperatures (orange circles, 23°C) or warmer temperatures (red squares, 28°C). Males (open symbols) and females (filled symbols) did not have significantly different WBFs after controlling for wing area and body size. Heavier flies tended to have faster WBFs (A; Table 2D), and flies with larger wings had slower WBFs (B; Table 2D) after statistically controlling for T_{dev} and T_{test}.

performance that we did not measure. Further analysis examining wing kinematics during hovering or high-powered flight might find that cold-reared flies adjust other parameters of wing-beat kinematics, such as stroke amplitude, the timing of wing rotation, the wing angle of attack, or the inclination of the stroke plane (Fry et al., 2005; Sane, 2003; Sane and Dickinson, 2001). Indeed, the larger wing areas could have costs in terms of flight performance, perhaps reducing maneuverability.

The relative contributions of genetic adaptation, plasticity and acclimatization, to the success of species distributed across thermal gradients are generally unknown. Genetic adaptation plays some role given that numerous studies have documented local genetic differences for insect populations across environmental gradients for traits such as wing size (Azevedo et al., 1998; David et al., 1994; Gilchrist and Huey, 2004; Loeschcke et al., 1999; Morin et al., 1999; Norry et al., 2001; Petavy et al., 1997; Starmer and Wolf, 1989) and chill tolerance (Ayrinhac et al., 2004). Developmental plasticity and/or acclimatization may also be important, especially given that local genetic adaptation may be hindered by extensive gene flow, particularly in mobile insect species with large geographic ranges, such as fruit flies.

Although several recent studies suggest that beneficial plasticity or acclimation may not be evolutionarily important, other evidence suggests that these processes can help organisms compensate for their environment (Barnes and Laurie-Ahlberg, 1986; Fischer et al., 2003; Li and Wang, 2005; Seebacher and Wilson, 2006; Wilson and Franklin, 1999). Beneficial plasticity may contribute to the ability of *D. melanogaster* to occupy a wide range of thermal environments. Ayrinhac and colleagues (Ayrinhac et al., 2004) showed that recovery of *D. melanogaster* from chill coma was due more to phenotypic plasticity (explaining 80% of the variability in this trait) than to genetic differences between high and low latitude populations (explaining 4% of the variability of this trait). For wing loading, phenotypic plasticity may also be more important than population level genetic differences for fruit flies living in cold environments. Gilchrist and Huey (Gilchrist and Huey, 2004) demonstrated that the wing area of *D. subobscura* increases as a result of both genetic and plastic responses to temperature. When populations along an altitudinal gradient were sampled and reared in common environments, a 1°C decrease in average yearly environmental temperature corresponded to a 0.03 mm^2 increase in wing area (based on populations from Denmark, 56°9′N; 10°13′E, average yearly temperature=7.5°C and Spain, 36°45′N; 4°25′W, average yearly temperature=16.4°C; data from females is used for all comparisons). The plasticity response appears to have a larger affect on wing size because every 1°C decrease in developmental temperature corresponded to about 0.11 mm^2 increase in wing area (based on a temperature range of 15–25°C). In our study, a 1°C decrease in the developmental temperature of *D. melanogaster* corresponded to approximately a 0.06 mm^2 increase in wing area (based on a temperature range of 15–28°C). The developmental plasticity response appears to be about 100–250% greater than the evolutionary response of wing area to temperature. This is a very rough estimate because we do not know the actual temperatures flies experience in their environment; nonetheless, the developmental response appears very important.

A large number of recent studies have rejected beneficial plasticity and acclimation (Blanckenhorn, 2000; Gibbs et al., 1998; Gibert et al., 2001; Huey et al., 1995; Leroi et al., 1994; Woods, 1999; Woods and Harrison, 2001; Zamudio et al., 1995), suggesting that these mechanisms are not evolutionarily significant ways for organisms to compensate for their environment. However, most of these studies have only tested performance at the organism's specific developmental temperature, addressing the question, 'do organisms perform better under the conditions that they are reared?' Our results suggest more studies should examine the possibility that plasticity has beneficial effects by pushing the thermal performance (or survival) envelope farther in the direction of the stress. The work of Overgaard and colleagues (Overgaard et al., 2008) also suggests this may be an important benefit of plasticity. In their study, *D. melanogaster* acclimated to 15°C temperatures were much more likely to survive long-term cold exposure than flies acclimated to 25°C. This benefit of plasticity may be particularly relevant to

insects, whose population sizes are strongly dependent on stochastic variation in weather (Price, 1997).

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