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EVOLUCIÓN TERMAL DEL POLIMORFISMO CROMOSÓMICO Y LA MORFOMETRÍA DEL ALA DE UNA POBLACIÓN EXPERIMENTAL DE *Drosophila subobscura*

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Los Doctores Mauro Santos y Antonio Fontdevila, Catedráticos del Departamento de Genética y Microbiología de la Facultad de Ciencias de la Universidad Autónoma de Barcelona,

Certifican que: Walkiria Janneth Céspedes Vigoya ha llevado a cabo bajo su dirección el trabajo de investigación realizado en el Departamento de Genética y de Microbiología de la Facultad de Ciencias de la Universidad Autónoma de Barcelona que ha llevado a la elaboración de la tesis Doctoral titulada: **Evolución Termal del Polimorfismo Cromosómico y la Morfometría del ala de una población experimental de *Drosophila subobscura***

Dr. Mauro Santos

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A los meus Amics

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ABREVIATURAS

Ba: Gen dominante *Bare* que determina el carácter del número de macroquetas. Número que normalmente está reducido en el mutante. Sin embargo las fosas de las cerdas están siempre presentes. Es letal en condición de homocigosis.

ch: Gen para el carácter de ojos color *cherry*

CS: *Centroid Size* – Tamaño del centroide

cu: Gen para el carácter de alas curvas (*curled*)

DA: *Directional Asymmetry* - Asimetría direccional

DI: *Developmental instability* – Inestabilidad del desarrollo

E: *Eastern*

FA: *Fluctuating Asymmetry* - Asimetría fluctuante

L1: Longitud del segmento basal o proximal de la vena longitudinal IV del ala

L2: Longitud del segmento distal de la vena longitudinal IV del ala

L-R: *Left-Right* – Izquierda - Derecha

N: *North*

PCs: Componentes principales

S: *South*

st: Ordenamiento cromosómico estándar

Va: Gen *Varicose,* gen dominante que determina el carácter carácter del engrosamiento irregular en la unión de las venas alares y la bifurcación en las venas de tamaño irregular corto. Es letal en condición de homocigosis.

W: *West*

WL: *Wing Length*– Longitud total del ala

WS: *Wing Shape* – forma del ala

1. INTRODUCCION

Diversas especies de *Drosophila* son colonizadoras, lo cual ha permitido utilizarlas como especies modélicas en el estudio de los importantes procesos evolutivos que acompañan la colonización. En el estudio de la colonización de América por *D. subobscura* se ha demostrado que la expansión ha sido muy rápida y que ha conducido al establecimiento del mismo tipo de clinas latitudinales para caracteres cromosómicos y morfológicos presentes en el Viejo Mundo (poblaciones paleárticas), lo que demuestra la plasticidad evolutiva de la especie a este nivel de variabilidad genética y la capacidad de su adaptación rápida según un gradiente latitudinal.

Las clinas latitudinales para ordenaciones cromosómicas aparecieron en tan sólo 3 años en Suramérica y 4 años en Norteamérica después del inicio de la colonización, aproximadamente hace 25 años. Las áreas colonizadas tienen una situación geográfica equivalente en ambos hemisferios, y las condiciones climáticas son paralelas a las del área de distribución en la región paleártica (Prevosti *et al*., 1988). Aunque los factores que afectan la distribución de una especie son diversos, lo anterior hace pensar que determinados factores ambientales, como la temperatura, pueden tener un papel muy importante en el cambio observado en los polimorfismos cromosómicos y en la morfología a lo largo del tiempo. Para intentar entender este particular proceso evolutivo que acompañó la colonización de *D. subobscura*, se hizo en el laboratorio un seguimiento cromosómico y morfométrico del ala de una población experimental, obtenida a partir de una población base muestreada en el epicentro de la colonización de América (Puerto Montt, Chile, 41º 28' S), que fue sometida a distintos regímenes térmicos: frió 13°, optimo 18° y cálido 22° C, y bajo un control exhaustivo de las condiciones de cultivo para poder dilucidar el posible papel de la temperatura como factor natural que determina la variación genética subyacente a esta evolución clinal.

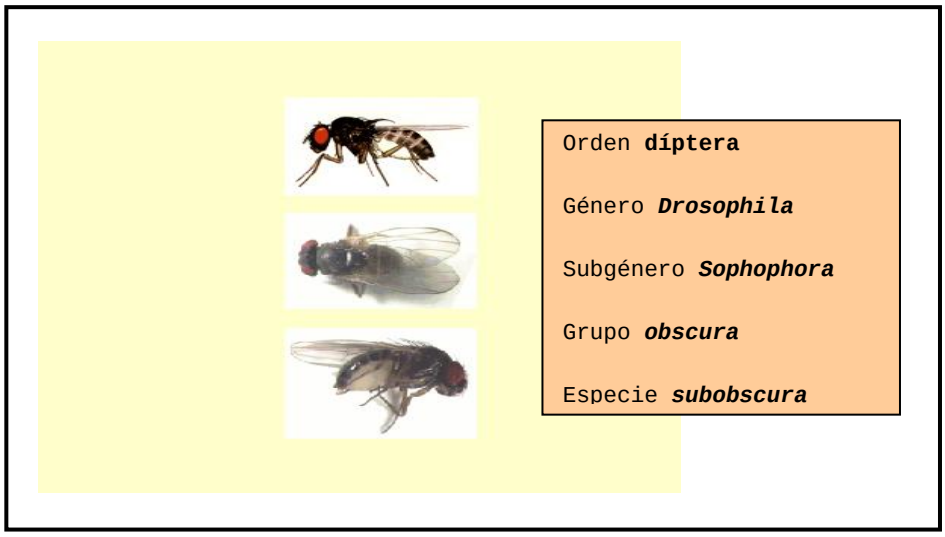
1.1. La especie *Drosophila subobscura*

Drosophila subobscura ha demostrado ser una especie de particular interés a la hora de estudiar la dinámica de la variación clinal y los efectos biológicos de la temperatura, primero porque existe una amplia información sobre su distribución geográfica; segundo, porque es especialmente rica en inversiones y, finalmente, porque una vez colonizó América demostró con gran rapidez un patrón de clinas latitudinales similares a las presentes en Europa.

Drosophila subobscura es una especie del grupo *obscura* que pertenece al subgénero *Sophophora* del género *Drosophila*, y el orden de los dípteros (ver figura 1). El subgénero *Sophophora* se divide a su vez en 7 grupos de especies: *melanogaster*, *obscura*, *saltans*,

willistoni, *dentisima*, *fima* y *dispar*. El grupo *obscura* cuenta actualmente con más de 35 especies. Sturtevant (1942) inicialmente dividió el grupo *obscura* en dos subgrupos (*obscura* y *affinis*) basándose en las siguientes características morfológicas: el número de las púas de los peines tarsales de los machos, la forma de los testículos y el número de agujas de quetas acrosticales. Y *Drosophila subobscura* fue incluida en el subgrupo *obscura*.

Figura 1: Vista dorsal y lateral de *Drosophila subobscura*



Posteriormente, Lakovaara y Saura (1982) y Beckenbach *et al*. (1993) consideraron que había suficientes diferencias entre los miembros neárticos y los paleárticos del subgrupo *obscura* como para dividirlos en dos subgrupos diferentes; así, propusieron una clasificación que organiza las especies del grupo *obscura* en 3 subgrupos: *pseudoobscura*, formado por unas 6 especies del oeste de Norteamérica; o*bscura*, constituida por unas 17 especies paleárticas; y *affinis*, que engloba especies neárticas junto con la especie paleártica *D. helvetica*. La única especie del grupo presente en el África tropical, *D. microlabis*, se mantuvo en un principio dentro del subgrupo *obscura*. Sin embargo, se descubrieron posteriormente en esta región 3 nuevas especies del grupo (Tsacas 1985; Cariou *et al*., 1988), y se propuso así la formación del subgrupo *microlabis* para incluir las 4 especies de África tropical del grupo.

Una de las últimas divisiones en subgrupos la propuso Barrio *et al*. (1994) que excluye 3 especies del subgrupo *obscura* (*D. subobscura, D. maderensis y D. guanche*) para formar el nuevo subgrupo *subobscura*. De esta manera, el grupo *obscura* quedaría dividido en 5 subgrupos; *affinis* y *pseudoobscura*, con especies típicas de Norteamérica; *obscura* y *subobscura*, típicas de Europa, y *microlabis*, típico de África (ver tabla 1). Cabe decir que los diversos estudios filogenéticos llevados a cabo con las especies del grupo *obscura* no han

aportado una información clara y definitiva respecto a su clasificación en subgrupos, lo cual aún hoy los investigadores no se han puesto de acuerdo ni sobre el número de subgrupos del grupo, ni sobre las especies que los integran (O'Grady 1999).

Tabla 1: División en subgrupos del grupo *obscura*

	Grupos	**Subgrupos**	**Especie**
Orden **Díptera**			
Género ***Drosophila***			
Subgénero ***Sophophora***	*melanosgaster*		
	saltans		
	willistoni		
	dentisima		
	fima		
	dispar		
	obscura	*afinis* (Sturtevant, 1942)	Neártica
		pseudoobscura (Lakovaara y Saura, 1982 y Beckenbach *et al*., 1993)	Neártica
		obscura (Sturtevant., 1942)	Paleártica
		subobscura (Barrio *et al*.,1994)	Paleártica
		microlabis (Tsacas, 1985; Cariou *et al*., 1988)	Etiópica

La primera cita de la especie *Drosophila subobscura* se debe a Collin (1936); posteriormente, otros autores hacen aportes nuevos para completar su descripción. Así, hoy sabemos que *Drosophila subobscura* es una especie polífaga de ecología mal conocida, aun a pesar de que parece una especie muy generalista. No se conoce exactamente en qué lugar de la naturaleza se reproduce y dónde pone los huevos; se han encontrado huevos de esta especie tanto en frutos como en plantas en descomposición, exudados vegetales y en hongos (Begon y Shorrocks 1978; Shorrocks 1982). Se cultiva fácilmente en el laboratorio y su ciclo biológico tarda unos 20 días en completarse a 18ºC, y a condiciones óptimas de densidad larval. Ha sido un organismo muy empleado en estudios genéticos, de ecología, fisiología, filogenia, comportamiento de insectos, y biología en general, por diversos grupos y científicos europeos. Es en general una especie muy bien estudiada desde el punto de vista de la genética evolutiva (Krimbas 1993; Powell 1997).

1.1.1 Distribución geográfica

El área de distribución de la especie en Europa se extiende prácticamente por todo el continente, incluso en las Islas Británicas y las del Mediterráneo (Islas Baleares, Córcega, Cerdeña, Sicilia, Creta, Chipre y las pequeñas Islas Italianas y Griegas), excepto en Islandia y parte norte y media de Escandinava. También se ha encontrado en diversas repúblicas de la antigua Unión Soviética (Sokolov y Dubinin 1941), la región iraniana del mar Caspio, Turquía, Líbano (Pipkin 1951), Israel y la zona costera del norte de África hasta el Sahara, en las Islas Canarias, Azores y Madeira (Krimbas 1993).

A finales de la década de los 70 e inicios de los 80, *D. subobscura* colonizó la costa oeste de América, tanto la del norte como del sur, y se extendió en este continente a lo largo de la costa ocupando las regiones correspondientes a las mismas latitudes (>15°) que ocupa en el continente europeo (ver figura 2).

Figura 2: Distribución geográfica de *Drosophila subobscura*[1]

En la naturaleza se encuentra cerca de las márgenes de los bosques de *Quercus*, *Abies*, *Pinus*, *Castanea*, *Ulmus*, y otros. De hecho, el límite norte de distribución de la especie en Europa coincide con el del *Quercus robur*. Sin embargo, a pesar de lo anterior, la especie parece más

[1] Mapa de la distribución geográfica de *Drosophila subobscura* tomado de: Tracking the genetic effects of global warming: *Drosophila* and other model systems. F. Rodríguez-Trelles, M. A. Rodríguez, and S. M. Scheiner. 1998. Conservation Ecology 2:2.

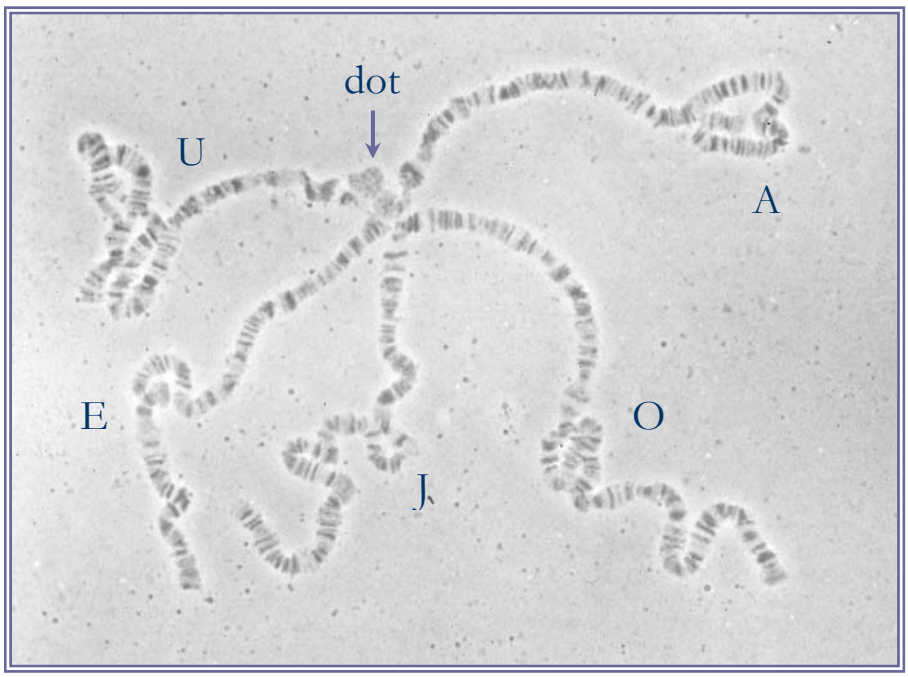
asociada a la actividad humana de lo que inicialmente se pensaba. Por otro lado, se ha demostrado en diversos estudios que se puede encontrar desde los niveles del mar hasta altitudes de más de 2000 metros sobre el nivel del mar.

La abundancia de *D. subobscura* en las diferentes zonas del área de distribución varia según si la región es central o marginal. En las regiones periféricas del área de distribución la especie se encuentra en un porcentaje más bajo del que se encuentra en la zona central de distribución, aunque es obvio que en los distintos países las estimas varían considerablemente, en parte por los procedimientos de muestreo usados para realizar dichas estimas. Así, por ejemplo, cuando los valores se miden en función del total de especies del grupo obscura, se encuentra que en el noreste y centro de Europa *Drosophila subobscura* es igual o menos abundante que *D. obscura*. En general, las poblaciones de las montañas balcánicas parecen ser ecológicamente centrales en comparación con las poblaciones del norte de Europa, África o el oriente medio.

1.1.2. Los cromosomas y sus inversiones

Drosophila subobscura tiene 6 pares de cromosomas, 5 cromosomas acrocéntricos denominados A, J, U, E y O, y un par puntiforme denominado dot (Mainx *et al*., 1953) (ver figura 3). El cromosoma A corresponde al cromosoma sexual y los cromosomas J, U, E y O son autosomas.

Figura 3: Preparación de los cromosomas politenicos de *Drosophila subobscura.*
(5 acrocéntricos y 1 puntiforme)



Los cromosomas acrocéntricos presentan un rico polimorfismo de inversiones en poblaciones naturales que ha sido muy estudiado. Uno de los primeros investigadores de estos polimorfismos fue Stumm-Zollinger (1953), quien estudió diversas poblaciones europeas y designó cada una de las diferentes inversiones identificadas. Sin embargo, su sistema de nomenclatura fue sustituido por otro que facilitaba la determinación numérica de las diferentes inversiones y ordenaciones cromosómicas, como se describe a continuación. Se han identificado más de 67 inversiones diferentes que forman más de 90 ordenamientos (o ordenamientos) cromosómicos resultantes de estas inversiones simples y de las combinaciones entre ellas (Krimbas y Loukas 1980; Krimbas 1992, 1993). Cada inversión es denominada con una letra que es la correspondiente a la del cromosoma en el que se encuentra, y con un número en subíndice que la identifica (ejemplo A_2), y que corresponde al orden temporal en que se fueron describiendo las distintas inversiones (Kunze-Muhl y Muller 1958).

Ahora bien, Mainx *et al*., (1953) con el fin de elaborar el mapa de *D. subobscura* utilizaron la cepa Kusnacht como fondo genético que permitió definir los puntos de rotura de las inversiones (ya que ésta es homocariotípica para todos los cromosomas y sus ordenamientos se definieron como estándar -Ast, Jst, Ust, Est y Ost-), y dividieron en 100 partes arbitrarias (pero de aproximadamente igual tamaño) al conjunto de cromosomas de la especie de *D. subobscura*; a cada parte la denominaron zona y comenzaron a enumerarlas por la región proximal del cromosoma A, de manera que la zona 99 es la región distal del cromosoma O y la zona 100 corresponde al cromosoma puntiforme o dot, lo que permite una correspondencia entre el número de partes y la longitud relativa de cada cromosoma. Por otro lado, cada zona se subdividió en 3, 4 ó 5 secciones que se designaron con las letras A, B, C, D y E; estas secciones se denominaron segmentos. Sin embargo, son Kunze -Muhl y Muller (1958) quienes hacen el mapa con mayor exactitud, manteniendo las 100 zonas y los 405 segmentos con que se ha dividido el conjunto de los cromosomas, entendiéndose así por 21A el segmento A de la zona 21, que corresponde al cromosoma J, y es uno de los puntos de rotura de una inversión. Finalmente, Krimbas (1993) publica un nuevo mapa, que actualiza el de Kunze-Muhl y Muller añadiendo los puntos de roturas de las inversiones descritas con posterioridad (ver figura 4).

Algunas inversiones se encuentran solas en el cromosoma, como por ejemplo la inversión A_2 (figura 5)[2], y otras pueden encontrarse solas o formando parte de ordenamientos complejos, como es el caso de O_7 (figura 6) que también se encuentra formando parte del ordenamiento O_{3+4+7} (figura 7). También se han encontrado algunas que sólo forman parte de un ordenamiento específico, como acostumbra pasar en las inversiones imbricadas que siempre se encuentran asociadas; por ejemplo la inversión E_9, que sólo está presente junto a las inversiones 1 y 2 en el ordenamiento E_{1+2+9} (figura 8) y nunca sola. Para indicar que las inversiones están imbricadas se subrayan los números de las inversiones que están imbricadas con una línea continua; por ejemplo $O_{\underline{3+4}}$ indica que las inversiones O_3 y O_4 son imbricadas (figura 9). En cambio, las inversiones en el mismo cromosoma pero no imbricadas se indican con una línea discontinua, como en el caso $O_{\underline{3+4}\ +\underline{7}}$, en el que se indica que las inversiones O_3 y O_4 están imbricadas, pero la inversión O_7 no lo está con cada una de las anteriores (figura 7). En las poblaciones naturales, se ha visto que las inversiones no imbricadas de un mismo cromosoma presentan un fuerte desequilibrio de ligamiento. Así, se ha observado que la ordenación O_7 presenta una baja frecuencia en las poblaciones naturales, en contraste con el ordenamiento $O_{\underline{3+4}\ +\underline{7}}$ que es más frecuente. Por otro lado, no hay datos claros que permitan afirmar que hay asociaciones entre inversiones de diferentes cromosomas, ni en poblaciones naturales ni de laboratorio (Sperlich y Feuerbach 1966).

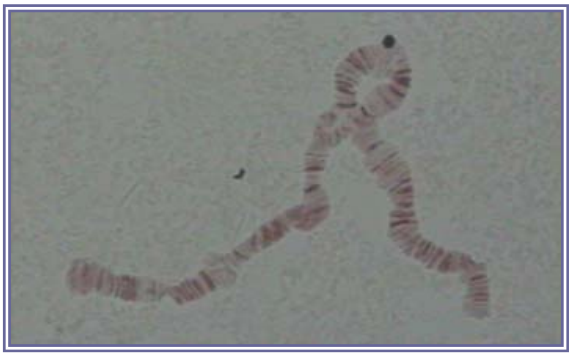


Figura 5: Inversión A_2

[2] Para definir los puntos de rotura de las inversiones, lo mejor es observar las inversiones sobre un ordenamiento estándar. Las inversiones que aquí se presentan están bajo un fondo genético de la cepa *ch-cu*, que es homocariotipica para todos los cromosomas, excepto para el cromosoma O que presenta la inversión 3+4.

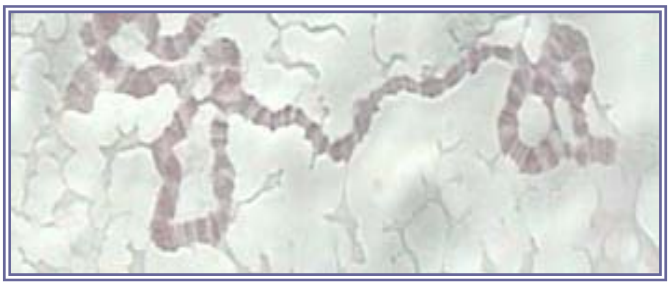


Figura 6[3]: Ordenamiento O_7

Figura 7[4]: Ordenamiento O_{3+4+7}

Figura 8: Ordenamiento E_{1+2+9}

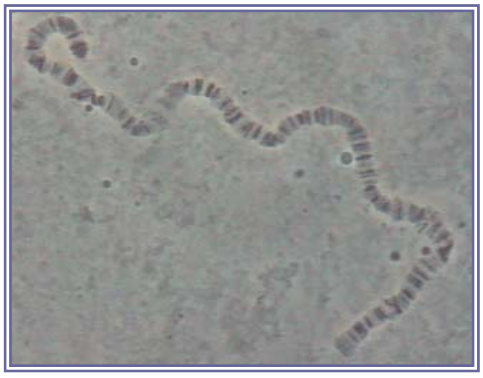


Figura 9: Ordenamiento O_{3+4}

1.1.3. Polimorfismos cromosómicos de *D. subobscura*

Una de las características importantes de *D. subobscura* como material de estudio en genética de poblaciones es que presenta un gran número de inversiones cromosómicas en las poblaciones naturales. Todos los cromosomas, excepto el puntiforme, presentan polimorfismos de inversiones. Estos polimorfismos han sido muy estudiados en Europa por científicos de diversos países (Krimbas y Loukas 1980; Krimbas 1993). Se han estudiado desde Finlandia

[3] En esta imagen se observa un doble bucle en la parte terminal del cromosoma que corresponde al fondo genético del cromosoma O de *ch-cu* que presenta la inversión 3+4.

[4] En esta imagen se observa la parte terminal del cromosoma O sin bucle porque al presentar la inversión 3+4 hibrida perfectamente con el fondo genético del cromosoma O de *ch-cu* que también es 3+4.

hasta Túnez y Egipto, incluyendo también algunas zonas de la antigua Unión Soviética. También se han estudiado poblaciones de Marruecos y de las islas Canarias, Madeira y Azores. En total se han analizado más de 130 poblaciones en la región paleártica. Todo esto ha permitido conocer la distribución de los diferentes ordenamientos y ver que en Europa algunas de estas distribuciones siguen unas clinas latitudinales. En algunas especies polimorficas de *Drosophila*, la frecuencia de las diferentes formas varía geográficamente. Cuando las frecuencias de las inversiones (carácter) experimentan un cambio gradual en una cierta dirección en función de la latitud (factor), se le denomina clina latitudinal (Huxley 1939, 1942; Haldane 1948; Endler 1977). Por ejemplo, en las poblaciones del norte predominan las inversiones estándar y su frecuencia va disminuyendo a medida que nos desplazamos hacia el sur. Otros ordenamientos son más frecuentes al sur de Europa y su frecuencia disminuye a medida que nos acercamos al norte. Algunas clinas no siguen estrictamente los meridianos. Por ejemplo, A_{st} y O_{st} presentan clinas NE-SW. Hay ordenamientos con clina E-W, como por ejemplo A_1. Otros ordenamientos son más frecuentes en un área determinada y su frecuencia disminuye a medida que se alejan de esta zona, como es U_{1+2+3} y $E_{1+2+9+12}$ en la región mediterránea, o J_{3+4} en la región mediterránea oriental, Turquía, Grecia y Cerdeña, o también U_{1+2+6} en la región de los Balcanes y Asia Menor, y E_{1+2+9} en los Balcanes.

Las clinas pueden formarse principalmente por un proceso de adaptación o como resultado de factores históricos. En el primer caso, el contenido genético de los diferentes ordenamientos cromosómicos daría una adaptación diferencial a los individuos frente a condiciones ambientales diferentes. Por ejemplo, en latitudes frías hay determinados ordenamientos que permiten una mejor adaptación de los individuos a estas condiciones que otros ordenamientos (A_{st}, J_{st}, U_{st}, E_{st} y O_{st}; Menozzi y Krimbas 1992; Orengo y Prevosti 1996), o a la inversa otros ordenamientos que permiten una mejor adaptación al calor (A_2, J_1, U_{1+2}, U_{1+2+8}, $E_{1+2+9+3}$, $E_{1+2+9+12}$, O_{3+4} y O_{3+4+8}; Menozzi y Krimbas 1992). En el segundo caso, las frecuencias de las inversiones dependerán de las circunstancias o el lugar en el que se origino la inversión; por ejemplo, la frecuencia de un determinado ordenamiento será más alta en la zona en que ésta se ha originado, e irá disminuyendo a medida que se alejen de esta zona. Inicialmente se discutía si las clinas latitudinales de algunos ordenamientos observados en la región paleártica eran debidas a un proceso adaptativo o a factores históricos. Sin embargo, la colonización de América por *Drosophila subobscura*, como se verá en la siguiente sección, ha permitido demostrar que las clinas latitudinales de los ordenamientos cromosómicos de esta especie obedecen a un proceso adaptativo, cuyo sistema de inversiones cromosómicas constituye un

mecanismo genético de adaptación rápida al ambiente, que le brinda una especial flexibilidad evolutiva.

1.2. La colonización de América por *D. subobscura*.

D. subobscura fue detectada por primera vez en 1978, en la población de Puerto Montt Chile (Lat. 41° 28' LS) (Brncic *et al*., 1981). Estudios posteriores muestran que la especie colonizó la zona central y sur del país, desde La Serena (Lat 29° 55' LS) hasta Punta Arenas (Lat 53° 10' LS) (Brncic y Budnik 1980; Brncic 1981; Budnik y Brncic 1982; Prevosti *et al*., 1983a 1989; Mestres *et al*., 1993) y se encontraron también individuos de esta especie en localidades de Argentina y Uruguay (López 1985; Goñi y Martínez 1995; Goñi *et al*., 1998). Su expansión por esta extensa área geográfica fue rápida pues tuvo lugar en pocos años.

En 1982 se detectó la especie en la localidad de Port Townsend, en el estado de Washington (EEUU) (Beckenbach y Prevosti 1986), y más tarde en diferentes localidades de la costa californiana (Ayala *et al*., 1989). Como sucedió en Suramérica, la especie no se había registrado tampoco antes en Norteamérica, como lo demuestran los análisis exhaustivos del polimorfismo cromosómico de algunas especies neárticas del subgrupo pseudoobscura hechas por Dobzhansky y colaboradores (Ayala *et al*., 1989). Actualmente la especie está distribuida por la costa oeste de Norteamérica, desde Ojai – California (Lat 38° 29' LN) hasta Puerto Ardí (Lat 50° 42' LN) en la Columbia Británica (Canadá) (Prevosti *et al*,. 1989; Noor *et al*., 1998; Huey *et al*., 2000). También se ha encontrado recientemente en las estribaciones rocosas junto a la ciudad del Provo en el estado de Utah (Noor *et al*., 1998).

El estudio del proceso de colonización de *D. subobscura* brinda una oportunidad única para entender la dinámica evolutiva de la especie. Este proceso de colonización se ha estudiado desde los puntos de vista biogeográfico, ecológico y genético (Brncic *et al*., 1981; Prevosti *et al*., 1983b, 1985, 1988, 1990; Balanyà *et al.*, 1994; Latorre *et al.*, 1986; Beckenbach y Prevosti 1986; Ayala *et al*., 1989; Rozas *et al*., 1990; Mestres *et al*., 1990, 1992, 1995, 2001; Rozas y Aguadé 1991; Pascual *et al*., 1993, 2001; Balanyà *et al*., 1994; Solé *et al*., 2000). El reconocimiento de que las áreas colonizadas por esta especie tienen una situación geográfica equivalente en ambos hemisferios, y las condiciones climáticas son paralelas a las del área de distribución en la región paleártica (Prevosti *et al*., 1988; Pergueroles *et al* 1995), y que el hecho de que en las poblaciones norteamericanas y suramericanas se encuentren los mismos ordenamientos cromosómicos da soporte a la idea de que ambos procesos de colonización no son independientes.

La rápida expansión de *D. subobscura* en las áreas colonizadas ha hecho que sea la especie más frecuente en algunas de las poblaciones analizadas. En los estudios realizados de los polimorfismos cromosómicos en América sólo se han detectado algunas de todas las inversiones conocidas en el Viejo Mundo, con un total de 18 ordenamientos cromosómicos de los 90 presentes en Europa: (2 para el cromosoma A; 2 para el cromosoma J; 5 para el cromosoma E; 3 para el cromosoma U y 6 para el cromosoma O (Brncic *et al*., 1981; Brncic *et al*., 1982; Balanyà *et al*., 2003; Krimbas 1992; Prevosti *et al*., 1988; Prevosti *et al*., 1989) (ver tabla 2 y figuras 10-22)[5]. Algunos de estos ordenamientos son muy frecuentes en Europa, otros son menos frecuentes o se encuentran sólo en determinadas zonas.

Por ejemplo, la inversión O_7 presente en Puerto Montt (Chile) está en muy baja frecuencia (0,7%), sin embargo este ordenamiento que también se encuentra periódicamente en el Viejo Mundo es probablemente el resultado de un evento de recombinación en el heterocariotipo O_{3+4+7}/O_{st} (Balanyà *et al*., 2003).

Por otro lado, en América han aparecido nuevas inversiones, como es el caso de E_{17} en las poblaciones de Santiago de Chile y Chillán (Chile) (Brncic *et al*., 1982; Prevosti *et al*., 1985) o de E_{18} en Eureka (California) (Pegueroles *et al.,* 1988), y que no se han vuelto a detectar en muestras posteriores. Como también ocurre con las inversiones halladas en Norteamérica E_{21} en Port Hardy (1994) y O_{26} en Centralia (1995) (Balanyà *et al.,* 2003). La hipótesis más aceptada actualmente es que un número de tal vez entre 4 a 150 individuos de Europa llegaron a América aportando una muestra significativa de los ordenamientos presentes en el Viejo Mundo (Brncic *et al*., 1982; Mestres *et al*., 1990; Pascual *et al*., 2001). Es por eso que en América, como consecuencia de un efecto fundador, se encuentran sólo algunos de los ordenamientos más frecuentes en Europa. Este efecto también aparece cuando se estudian otros marcadores de la variabilidad genética como las aloenzimas y los microsatélites. Algunos estudios genéticos que se basan en el polimorfismo enzimático muestran que en América se encuentran algunos de los alelos más frecuentes en Europa, pero no aquellos muy poco frecuentes (Balanyà *et al*., 1994). En los alelos de los loci microsatélites también se ha observado una disminución en el número de estos entre Europa y América. En Europa se

[5] En este listado de figuras aparecen únicamente 13 ordenamientos de los 18 existentes, los 5 restantes aparecen en las figuras referenciadas en la sección 1.1.2 (Los cromosomas y sus inversiones). E igualmente, las inversiones que aquí se muestran están bajo el fondo genético de la cepa *ch-cu*.

encuentran entre 18 y 36 alelos por cada locus; en cambio, en Norteamérica el número oscila entre 4 a 8, es decir, hay una clara reducción en el numero de alelos (Pascual *et al*., 2001).

También se ha observado en las poblaciones colonizadas que la inversión O_5 presenta una asociación compleja con un gen letal (Ayala, Serra y Prevosti 1989; Prevosti 1989; Mestres *et al*., 1990, 1992, 1995; Solé *et al*., 2000). Las pruebas de alelismo indican que se trata del mismo gen letal para ambas poblaciones, lo que refuerza el origen común de los colonizadores de Norteamérica y Suramérica. Probablemente un individuo de la muestra de colonizadores era portador de una inversión O_5 con el gen letal (Mestres y Serra 1995). Se ha intentado descubrir cuál era la población europea de origen buscando aquella que contenga un O_5 con un letal alélico como el de las poblaciones americanas. Hasta hora no se ha encontrado ninguno que cumpla esta condición; a pesar de que se han encontrado inversiones O_5 en algunas poblaciones europeas muestreadas, unas no eran portadoras de ningún gen letal y otras portaban un letal que no era alelico con el O_5 americano (Mestres *et al*., 1992; Zivanovic y Mestres 2000).

Tres años después del primer registro de *D. subobscura* en Suramérica, y cuatro años después en Norteamérica, se detectó la presencia de clinas latitudinales para algunos ordenamientos cromosómicos en las poblaciones americanas (Prevosti *et al*., 1985, 1988). Estas clinas latitudinales tienen el mismo sentido en las dos áreas colonizadas y son análogas a las que se habían observado en Europa (Ayala, Serra y Prevosti 1989; Brncic *et al*., 1981; Prevosti *et al*., 1988, 1989, 1990), excepto para la inversión O_5 que es rara, está distribuida irregularmente en la región paleártica y su frecuencia no varía con la latitud (Krimbas y Loukas 1980; Krimbas 1992, 1993; Zivanovic y Mestres 2000). Sin embargo, en Norteamérica y Suramérica esta inversión desarrolló rápidamente clinas latitudinales significativas.

En general, los datos obtenidos en los últimos muestreos, que en conjunto abarcan más de dos décadas en Norteamérica y casi tres décadas en Suramérica, demuestran que la evolución de las clinas parece haberse estabilizado según un modelo convergente con el observado en la región paleártica (Solé *et al*., 2002), en el que las correlaciones de las frecuencias de cada una de las ordenaciones cromosómicas respecto a la latitud continúan presentando el mismo signo que en la región paleártica, aunque no alcanzan los valores observados en el Viejo Mundo (Balanyà *et al*., 2003). Así, el establecimiento de dichas clinas y su equivalencia con las europeas, refuerzan claramente la hipótesis adaptativa y sugieren una evolución rápida, continua y predecible de los polimorfismos en esta especie.

Tabla 2: Ordenaciones cromosómicas en América.

Crm[1]	**Polimf**[2]	**Ubicación**[3]	**Denominación**[4]	**%crm**[5]
A	A_{st}			
	A_2	Inversión A_2 8c/d - 12c/11B	Aldo	31
J	J_{st}			
	J_1	Inversión J_1 22B/22C- 25A (2)/25D	Ingrid	13,3
U	U_{st}			
	U_{1+2}	Inversión U_1 39D/40A - 45E/46A Inversión U_2 45E/46A - 51C/15D Inversiones generalmente asociadas	Urs Umberto	31,8 33,3
	U_{1+2+8}	Inversión U_8 40A/51C- 44D/45A Inversión U_8 imbricada y sobrepuesta parcialmente a U_{1+2}		25,3
E	E_{st}			
	E_{1+2}	Inversión E_1 58D/59ª Inversión E_2 58D/62D - 64B/64C Inversiones imbricadas y sobrepuestas	Edda Eleonore	26,9
	E_{1+2+9}	Inversión E_9 58D/68B - 64B/64C Inversión E_9 imbricada a E_{1+2}	Ernst	43,9
	$E_{1+2+9+3}$	Inversión E_3 58D/68B – 64C/62D Inversión E_3 imbricada a E_{1+2+9}		
	$E_{1+2+9+12}$	Inversión E_{12} 67B/67A - 61D/61C Inversión E_{12} imbricada y sobrepuesta a E_{1+2+9}		
	E_{17}	Inversión E_{17} 54D/64B – 64C	única en Chile	
O	O_{st}			
	O_{3+4}	Inversión O_3 91B/91C – 94E/95A Inversión O_4 94D/94E – 98C/98D Inversiones Imbricadas y sobrepuestas	Orson Ottilie	11,8 25,2
	O_{3+4+8}	Inversión O_8 90D/91A - 94A/94B Inversión Sobrepuesta a O_{3+4}	Olga	26,6
	O_{3+4+7}	Inversión O_7 77B/77C – 85E Inversión Casi siempre asociada a O_{3+4}	Oskar	31,6
	O_{3+4+2}	Inversión O_2 85C/85D y 89B/89C Inversión O_2 imbricada a O_{3+4}	Ottokar	18,3
	O_{3+4+22}*	Inversión O_{22} 83C/84Aª y 87A/87B Inversión O_{22} independiente y asociada a O_{3+4}		14,9

1: Crm = Cromosoma **2**: Polimf = polimorfismo **3**: Según mapa de Kunze-Muhl y Muller (1958) **4**: Según Stumm-Zollinger (1953) **5**: Porcentaje de la longitud del cromosoma que comprende la inversión

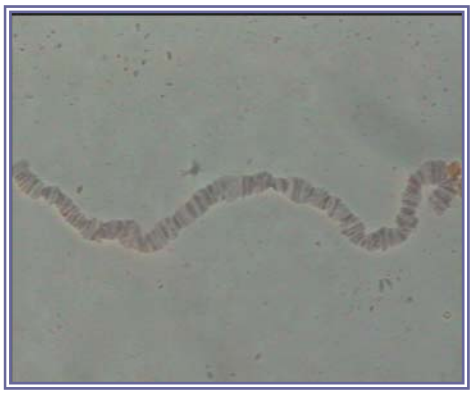


Figura 10: Ordenamiento A_{st}

Figura 11: Ordenamiento J_{st}

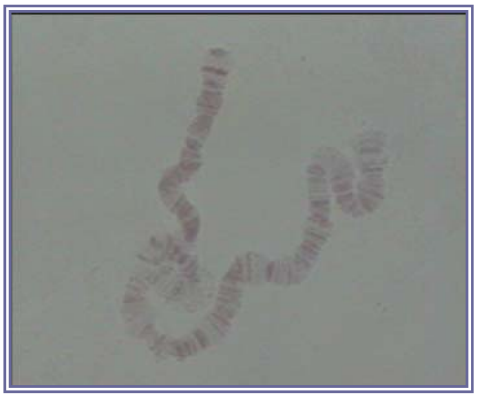


Figura 12: Ordenamiento J_1

Figura 13: Ordenamiento U_{st}

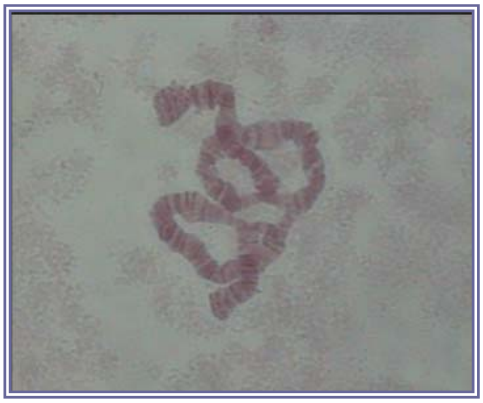


Figura 14: Ordenamiento U_{1+2}

Figura 15: Ordenamiento U_{1+2+8}

Figura 16: Ordenamiento E_{st}

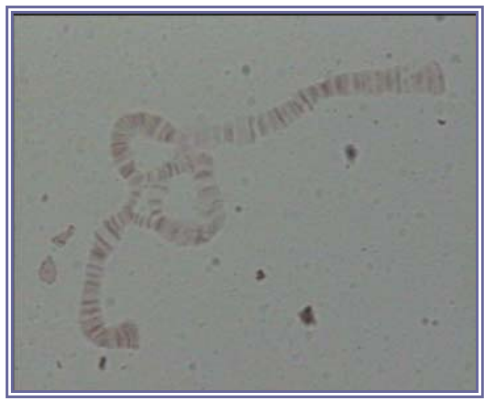


Figura 17: Ordenamiento $E_{1+2+9+3}$

Figura 18: Ordenamiento $E_{1+2+9+12}$

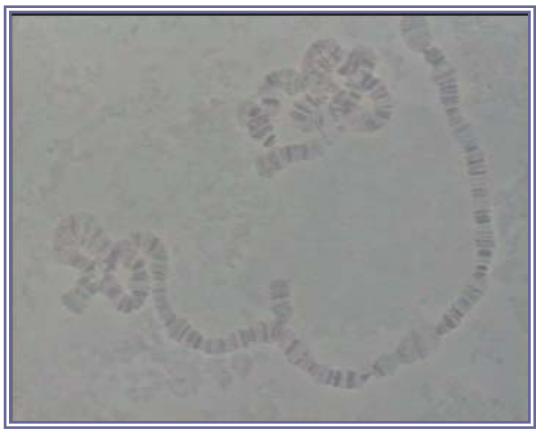


Figura 19: Ordenamiento O_{st}

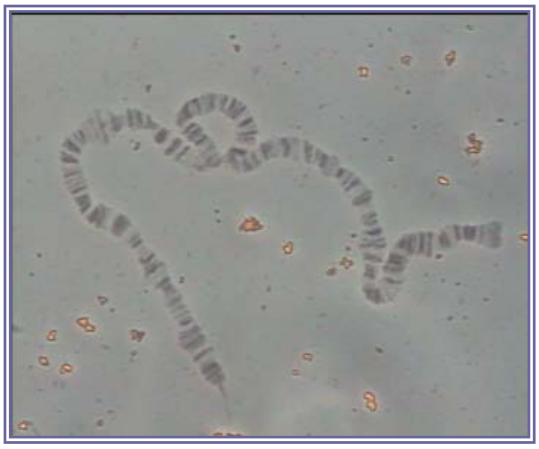


Figura 20: Ordenamiento O_{3+4+2}

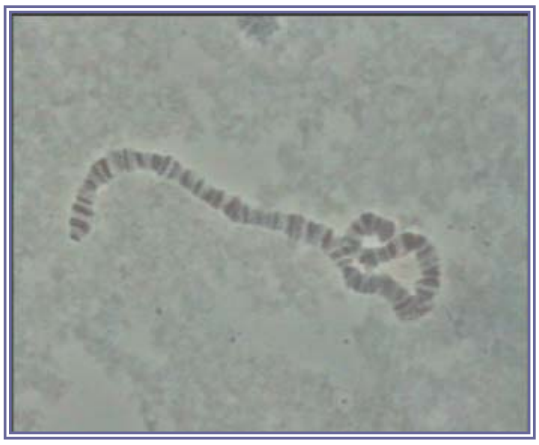


Figura 21: Ordenamiento O_{3+4+8}

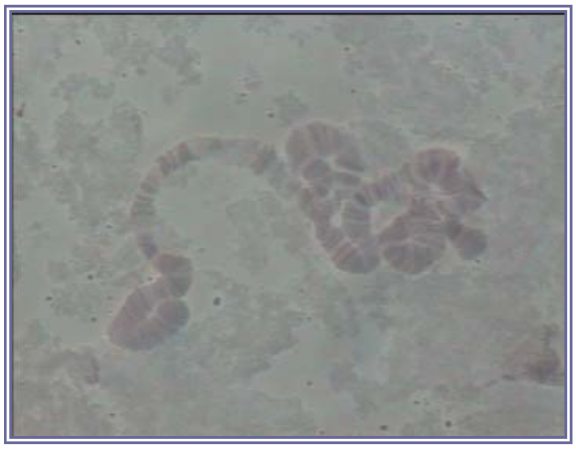


Figura 22: Ordenamiento O_5

1.3. Carácter adaptativo de las inversiones cromosómicas

La presencia de genes complejos en los segmentos invertidos de los cromosomas, que son adaptativos para diferentes condiciones ambientales, podría explicar el rápido efecto de la selección natural y el establecimiento de las clinas en las poblaciones naturales de *D. subobscura* (Prevosti *et al*., 1985).

Hay dos teorías que pueden explicar el carácter adaptativo de las inversiones cromosómicas. En primer lugar, las inversiones pueden producir un efecto de posición sobre algunas secuencias, o sea, provocan un cambio en la localización física de algunos genes que podría ser beneficioso para los individuos portadores (Sperlich 1963). Este cambio de posición puede alterar la expresión de algunos genes (Puig *et al.*, 2004) ya que permite cambiar su posición respecto a sus secuencias reguladoras y se espera que sea más importante en las secuencias próximas a los puntos de corte.

En segundo lugar, las inversiones reducen la recombinación dentro de la región incluida en la inversión de los heterocariotipos. En muchas especies como en *Drosophila* (White 1973; Sorsa 1988) los machos no recombinan y, por otro lado, las hembras poseen un mecanismo ordenado de meiosis que hace que los cromosomas aberrantes, producidos por el entrecruzamiento durante el emparejamiento de las regiones homologas de las inversiones en la recombinación, acaben siempre en los corpúsculos polares (White 1973; Roberts 1976; Coyne *et al.*, 1993), evitando con esto el descenso de la fertilidad y la selección negativa. De esta manera dentro de las inversiones se pueden mantener ciertas combinaciones de alelos favorables, ya que están protegidos de la recombinación (Krimbas y Powell 1992).

Finalmente, un punto importante en el mantenimiento de un polimorfismo estable de las inversiones, es las relaciones entre los loci atrapados por la inversión respecto a la aptitud (*fitness)*, que pueden ser o bien aditivas, en las que no hay interacción entre loci y es extremadamente difícil alcanzar un polimorfismo estable, y si lo alcanza es porque la inversión puede ser constantemente favorable; debido a un efecto de posición o a la ausencia total de recombinación que erosione su ventaja inicial (Ohta y Kojima 1968; Nei *et al.*, 1967; Cook y Nassar 1972); o bien epistáticas, que suponen interacciones entre loci, que pueden constituir complejos génicos coadaptados (Nei 1967; Wasserman 1968; Feldman 1972; Charlesworth y Charlesworth 1973; Charlesworth 1974; Deakin 1972; Deakin y Teague 1974¸Teague y Deakin 1976; Feldman *et al*., 1980) y cuyas frecuencias varían según las presiones selectivas existentes en el ambiente en el que se desarrollan (Hartl 1977; Wallace 1991).

1.4. Variación del polimorfismo cromosómico en *D. subobscura*.

Existen evidencias del carácter adaptativo de las inversiones cromosómicas que favorecen su mantenimiento en las poblaciones naturales. En primer lugar, se han llevado a cabo estudios con cajas de poblaciones en diferentes especies de *Drosophila* y los resultados derivados de

estos estudios sugieren que las inversiones se mantienen en las poblaciones naturales por una selección equilibradora debida a la superioridad de los heterocariotipos (Sperlich y Pfriem 1986; Powell 1997). En segundo lugar, se han descrito variaciones temporales y espaciales regulares en las frecuencias de las inversiones en poblaciones naturales. Algunas especies de *Drosophila* muestran cambios estacionales recurrentes o cíclicos de las frecuencias relativas de diferentes ordenaciones cromosómicas (Fontdevila *et al*., 1983; Sperlich y Pfriem 1986; Rodríguez-Trellez *et al*., 1996), y en algunos casos se ha relacionado la distribución de las frecuencias de ordenaciones cromosómicas con factores ambientales. En las poblaciones de *Drosophila melanogaster*, por ejemplo, los dos hemisferios (norte y sur) presentan una distribución similar de las frecuencias de 4 inversiones siguiendo clinas latitudinales (Lemeunier y Aulard 1992); en *D. robusta* se han formado clinas altitudinales similares en diferentes áreas montañosas (Carson 1958; Etges 1984; Levitan 1992; 2001) y en *D. pseudoobscura* se han observado cambios que siguen un patrón de variación estacional muy claro (Dobzhansky 1943; Wright y Dobzhansky 1943; Dobzhansky 1948).

En *D. subobscura* los datos obtenidos por diversos autores (Burla y Gotz 1965; De Frutos y Prevosti 1984; Burla *et al*., 1986; Gosteli 1990; Mestres *et al*., 1994) muestran que el polimorfismo de inversiones varía considerablemente a lo largo del tiempo. Sin embargo, los cambios observados no siguen un patrón de variación estacional tan claro, como lo demuestran los estudios realizados durante varios años en una población de Viena, en la que no se detectaron cambios estaciónales cíclicos para diversos ordenamientos cromosómicos (Kunze-Muhl *et al*., 1958; Sperlich y Feuerbach 1996). Asimismo, podemos citar los estudios realizados en poblaciones europeas, como en la población griega de Mt. Parnes (Krimbas 1967), donde se analizaron los cambios en las frecuencias de algunos ordenamientos durante dos años consecutivos y se encontraron algunos cambios únicamente en el primer año, aunque éstos no se correspondían con los esperados según las clinas latitudinales encontradas en esta especie. En la población gallega de El Pedroso, al analizar el polimorfismo del cromosoma O durante 4 años se encontró que algunos ordenamientos presentaban estacionalidad (Fontdevila *et al*., 1983). Por ejemplo, se observó un aumento en el ordenamiento O_{3+4+7} durante el tiempo de estudio, contrario a lo que se detectó para el ordenamiento O_{st}, cuya frecuencia disminuyó en otoño y primavera. Posteriormente, entre 1988 y 1991 se volvió a estudiar la variación estacional de este polimorfismo en el cromosoma O (Rodríguez-Trelles *et al*., 1996, 1998; Rodríguez – Trelles y Rodríguez 1998) y se detectó nuevamente la estacionalidad de algunos ordenamientos como O_{3+4+7} y O_{st}, en el mismo sentido que en el primer trabajo. Este cambio estacional presenta un comportamiento acorde con la distribución geográfica clinal de estos

ordenamientos. Es decir, O_{st}, que es muy frecuente en las poblaciones del norte, presentó mínima frecuencia durante el estudio, mientras que O_{3+4+7}, típico de las poblaciones del sur, tuvo un máximo. Las frecuencias de estos ordenamientos demostraron también estar fuertemente asociados a factores climáticos como la temperatura máxima diaria y la humedad relativa. Finalmente, De Frutos y Prevosti (1984), analizaron y compararon en la población de Barcelona diversas muestras obtenidas a lo largo de un año y no detectaron variación en las frecuencias de los ordenamientos cromosómicos de los cromosomas A, E, y O, pero sí en los del J y U; los ordenamientos J_1 y U_{1+2+8} presentaban ambos un máximo en los meses de junio y febrero, y un mínimo en el de septiembre, pero los datos no fueron suficientes como para comprobar si seguía un modelo cíclico.

Otros estudios similares de la variación del polimorfismo cromosómico se han llevado a cabo también pero en relación con la altitud, y aunque éstos son pocos, demuestran que hay diferencias significativas. Es el caso del trabajo de Burla *et al*. (1986) quienes encontraron diferencias en muestras capturadas entre 600 y 1900 metros sobre el nivel del mar en la región suiza de Valais. Estas diferencias afectaban sobre todo al cromosoma J, el cual presentaba una variación con la altitud concordante con la variación latitudinal observada para éste cromosoma. Se hizo otro estudio, en este mismo sentido, con 4 poblaciones de un valle próximo a Santiago de Chile entre agosto y septiembre de 1985. Las poblaciones analizadas se localizaban a una altura entre el nivel del mar y los 1900 metros sobre el nivel del mar. Al comparar el polimorfismo cromosómico entre estas poblaciones se encontraron diferencias significativas en los ordenamientos E_{st}, E_{1+2+9}, $E_{1+2+9+12}$, O_{3+4+2}, U_{st} y U_{1+2}, y a pesar de que no se observó una clina altitudinal, ciertos ordenamientos demostraron seguir clinas latitudinales (Brncic y Budnik 1987). Muy contrarios son los resultados obtenidos por Martínez - Sebastián *et al*. (1984), quienes estudiaron tres poblaciones de la Sierra de Gudar, en la provincia de Teruel, situadas a diferentes altitudes, y no detectaron diferencias significativas entre ellas, a pesar de que había claras diferencias ecológicas y climáticas entre las diferentes localidades.

Por otro lado, se han obtenido resultados más consistentes en estudios de los cambios temporales a largo plazo del polimorfismo cromosómico. Uno de los primeros estudios se realizó en una población cercana a Zurich (Gosteli 1990), en el que se compararon muestras obtenidas entre los años 1986 y 1987 con otras que ya habían sido analizadas con anterioridad (1963, 1964 y 1984) por otros autores (Burla y Gozt 1965). En el estudio se encontraron diferencias entre las muestras, los ordenamientos A_{st}, J_{st}, U_{st} y O_{st} disminuyeron, mientras que J_1, U_{1+2}, O_{3+4} y O_{3+4+8} aumentaron. En el estudio de la población de El Pedroso (Fontdevila *et*

al., 1983; Rodríguez-Trelles *et al*., 1996, 1998; Rodríguez-Trelles y Rodríguez 1998) al comparar los datos obtenidos entre los años 1976 - 1980 con los de los años 1988 - 1991 se aprecia una cierta disminución de los ordenamientos O_{st} y O_{3+4+8} y un aumento significativo del ordenamiento O_{3+4}. Orengo y Prevosti (1996) analizaron los cambios en el polimorfismo cromosómico en un intervalo de 29 años de una población de Barcelona obtenida en el observatorio Fabra en noviembre de 1988 y 1989, y la compararon con cinco muestras reportadas en los años previos por diferentes autores (Prevosti 1964; De Frutos y Prevosti 1984). Los resultados obtenidos muestran que en general los ordenamientos estándar, típicos de las poblaciones del norte, disminuyen, mientras que otros ordenamientos más complejos, típicos de las poblaciones más del sur, han incrementado su frecuencia. Igualmente, se encontró una cierta correlación entre la temperatura y la frecuencia de ciertos ordenamientos que en algunos casos era significativa. Esto indicaría que las clinas latitudinales para el polimorfismo cromosómico son debidas a factores ambientales (Prevosti 1985, 1988, 1990; Menozzi y Krimbas 1992), y que su variación geográfica adaptativa podría estar relacionada con diversos factores ambientales y ecológicos, como por ejemplo la temperatura.

La aparición de las clinas latitudinales en las frecuencias de algunos polimorfismos cromosómicos en las poblaciones colonizadas por *D. subobscura*, equivalentes a las existentes en la región paleártica de origen, muestran el carácter rápido y predecible de la evolución de dicho polimorfismo. Asimismo, el estudio de sus cambios a largo plazo permitió también analizar el posible efecto del cambio climático global del planeta sobre la estructura genética de las poblaciones. En este sentido, Solé *et al.* (2002) encontraron que las frecuencias de muchas ordenaciones cromosómicas han variado de forma sistemática y significativa durante los últimos 26 a 35 años en muchas poblaciones europeas. La frecuencia de las ordenaciones típicas de latitudes más cálidas (sur) ha aumentado en todas las poblaciones estudiadas, mientras que la frecuencia de las ordenaciones típicas de latitudes más frías (norte) ha disminuido, estos cambios podrían explicarse por una variación en la temperatura o por otros factores ambientales relacionados con la latitud, que hacen la composición cromosómica de esta especie más característica de ambientes cálidos. Cabe destacar también que las frecuencias de cinco ordenaciones de Suramérica han experimentado cambios direccionales sistemáticos a escala continental, en la que la frecuencia de los ordenamientos U_{st} y E_{1+2+9}, disminuye, mientras que la de los ordenamientos O_{3+4}, $E_{1+2+9+12}$ y U_{1+2+8} aumentan. Estos cambios observados son equivalentes a los detectados en 13 poblaciones europeas en un periodo no inferior a dos décadas y corresponden a lo que se esperarían si el polimorfismo cromosómico

de la especie se estuviera adaptando al incremento de la temperatura ocasionado por el cambio climático global del planeta (Balanyà *et al*., 2003).

Todas estas observaciones, aparte de ser evidencias de la existencia de presiones selectivas sobre las inversiones, demuestran que los polimorfismos de inversiones en *D. subobscura* constituyen un mecanismo genético de adaptación rápida al ambiente, que da a la especie flexibilidad adaptativa.

1.4.1. Clinas latitudinales de tamaño corporal en *D. subobscura*

Como ya se mencionó anteriormente, las clinas pueden ser el producto de un proceso adaptativo o el resultado de factores históricos, sin embargo las correlaciones positivas encontradas para los caracteres (inversiones o tamaño corporal) respecto a la latitud y más exactamente en relación con la temperatura, nos sugiere que éstas son más una respuesta adaptativa a la variación geográfica (Endler 1977; Partridge y French 1996).

De acuerdo a lo observado para las poblaciones paleárticas que muestran un incremento clinal del tamaño corporal con la latitud (Prevosti 1955; Misra y Reeve 1964; Pfriem 1983), junto a la aparente naturaleza adaptativa de las clinas latitudinales para tamaño corporal, se esperaría que clinas similares fueran detectadas en el continente americano. Sin embargo, en los estudios realizados por algunos autores (Budnick *et al*., 1991; Pergueroles *et al*., 1995) una década después de la colonización de América por *D. subobscura*, tanto en Norteamérica como en Suramérica, no se encontraron evidencias de dichas clinas. Hizo falta una década más para que los estudios realizados por Huey *et al*. (2000), en los que se examinaban las poblaciones de Norteamérica de 11 localidades del oeste y las contrastaban con las poblaciones de l0 localidades de Europa continental, demostraran el mismo patrón de clinas de los estudios previos en la región paleártica. Aunque las clinas en Norteamérica y Europa involucraban diferentes cambios en la longitud relativa de distintas secciones del ala, con un incremento en la longitud del ala en Europa causado por un alargamiento de la porción basal de la vena IV, mientras que el incremento en Norteamérica era causado por un relativo alargamiento de la porción distal de la vena IV. En este sentido, el trabajo de Birdsall *et al*. (2000) sugiere que estas diferencias podrían deberse a la base celular de las clinas, sin embargo los estudios de Calboli *et al*. (2003a) demuestran todo lo contrario, ya que la base celular de la clina del tamaño del cuerpo en *D. subobscura* en Norteamérica, es diferente de las clinas de Europa y Suramérica, puesto que en Norteamérica están basadas en la variación latitudinal en el área celular, mientras que en Suramérica y Europa la variación latitudinal es en número celular.

Lo anterior demuestra que la evolución de la longitud del ala es previsible, aunque su respuesta no es tan rápida como la encontrada para las clinas de las inversiones cromosómicas y, además, refuerza la idea del carácter adaptativo de las clinas latitudinales para tamaño corporal (las dimensiones del ala son usadas como un índice del tamaño corporal: Robertson y Revee 1952; Misra y Revee 1964; Anderson 1966; Sokoloff 1966; Cowley y Atchley 1990).

1.4.2. Relación entre la temperatura y las características morfométricas

Algunas características morfométricas están positivamente correlacionadas con la temperatura u otras variables ambientales en varias especies de *Drosophila*. En los análisis reportados por diferentes autores, la variabilidad genética de algunas características morfométricas se ha demostrado tanto en la naturaleza como en poblaciones de laboratorio. Por ejemplo, en *D. melanosgaster* y en *D. simulans* el peso de los adultos y el número de ovarios en las hembras incrementa linealmente con la latitud (David y Bocquet 1975). En las poblaciones naturales de *D. robusta* también se observan clinas latitudinales y correlacionadas positivamente con la temperatura para algunas características morfométricas (Stalker y Carson 1947, 1948). Los experimentos en cajas de poblaciones mantenidas a diferentes temperaturas han mostrado que las características biométricas de algunas especies de *Drosophila* pueden cambiar significativamente en pocos años. Así un stock criado a baja temperatura produce individuos genéticamente más grandes que un stock criado a temperatura alta donde los individuos son pequeños (*D. pseudoobscura*: Druger 1962; Anderson 1966, 1973; Powell 1974; *D. melanogaster*: Cavicchi *et al*., 1978; Cavicchi *et al*., 1985; Huey *et al*., 1991; Partridge *et al*., 1994; James y Partridge 1995).

Aunque los mecanismos selectivos que favorecen el tamaño del ala grande en latitudes altas en algunos ectodermos es aun desconocido, la hipótesis más generalizada es la respuesta adaptativa a la temperatura (Partridge y French 1996), que se refuerza con la respuesta rápida del tamaño corporal demostrada por algunas especies de *Drosophila* a los cambios ambientales, principalmente a la temperatura. Por ejemplo, en *D. melanogaster* se han reportado clinas latitudinales en tamaño del cuerpo como evidencias de la adaptación a la selección impuesta por la temperatura (Norteamérica: Coyne y Beecham 1987; Capy *et al.,* 1993; en oeste de Europa y África: Capy *et al*., 1993; Gilchrist *et al.,* 2000; este de Europa y Asia: Imasheva *et al,* 1994; Suramérica: Van't Land *et al.,* 1995; Gilchrist *et al.,* 2000 y, Australia: James *et al.,* 1995).

Los estudios realizados por Pergueroles *et al.* (1995), en los que se analizan la diferenciación latitudinal en las características cuantitativas de las poblaciones paleárticas de *D. subobscura* y de *D. obscura*, dos especies estrechamente relacionadas, encontraron que ambas especies tienen una respuesta paralela con la latitud. Además, es probable que las clinas observadas sean adaptativas y probablemente relacionadas con el clima, ya que tienen un patrón de concordancia con la variación geográfica. Una observación de una correlación paralela (tamaño con latitud) entre dos o más especies diferentes implica que las dos especies están afectadas por factores ambientales similares y los patrones reflejan el impacto de la selección natural (Endler 1986).

Los estudios realizados por Gilchrist *et al.* (2001) sobre las clinas de tamaño del ala de *D. subobscura*, comparando el patrón latitudinal y la dinámica evolutiva de selección entre muestras de poblaciones norteamericanas tomadas en 1986 y 1997, contrastadas con muestras de poblaciones europeas tomadas en 1998, sugieren que las clinas de tamaño desarrolladas en Norteamérica representan una adaptación debida a la selección impuesta por la temperatura, en la que se refleja la selección del tamaño más grande del cuerpo a temperaturas medias más frías asociadas con latitudes más altas.

1.4.3. Relación entre el polimorfismo cromosómico y el tamaño del ala

Las clinas latitudinales tanto del polimorfismo cromosómico como del tamaño del ala en *D. subobscura* sugieren que podrían ser señales del mismo fenómeno, o lo que es lo mismo, la acción de la selección natural en el tamaño del cuerpo (nivel fenotípico) podría explicar las observaciones en la variación en el polimorfismo cromosómico (nivel genotípico) de esta especie (Orengo y Prevosti 2002). Sin embargo, el polimorfismo cromosómico de *D. subobscura* es muy complejo, ya que los 5 cromosomas acrocéntricos son polimórficos y un gran número de ordenamientos cromosómicos pueden coexistir en una población natural, lo que complica mucho la relación entre polimorfismo de inversiones y tamaño corporal.

Prevosti (1967) demostró en experimentos de selección artificial que las alas cortas en *D. subobscura* muestran una clara tendencia hacia la homocigosis de los ordenamientos cromosómicos típicos de poblaciones del sur, las cuales tienen una media más pequeña del tamaño corporal. Además, la mayoría de las líneas seleccionadas para alas largas mantenían dos ordenamientos cromosómicos con frecuencias oscilando alrededor del 50%. Estos ordenamientos se fijaron para un orden complejo de líneas de alas cortas y ordenamientos estándar, típicos de poblaciones del norte con un tamaño medio más grande. Krimbas (1967)

observó una relación entre el cariotipo de machos para el cromosoma A (=X) y el tamaño del cuerpo. Este autor observó, en una población en Grecia, que los machos portadores del ordenamiento A_{st} eran más grandes que los portadores de A_2. En los estudios llevados a cabo por Orengo y Prevosti (2002) en los que se analizaba este tipo de relación entre los machos de dos muestras de poblaciones naturales de *D. subobscura,* ya analizadas para cambios a largo plazo en el polimorfismo de inversión (Orengo y Prevosti 1996) y para la heredabilidad del tamaño del ala (Orengo y Prevosti 1999), se detectó una asociación entre los ordenamientos cromosómicos y el tamaño para 4 de los 5 cromosomas polimórficos (excepto el J), indicando que en general los ordenamientos estándar determinan alas más grandes y demostrando así la idea de una relación entre el polimorfismo y el tamaño corporal.

La frecuencia de los ordenamientos estándar varía en la misma dirección en todos los cromosomas (Krimbas 1992), y un incremento en su frecuencia es paralelo a un incremento del tamaño del cuerpo. Los ordenamientos estándar son los que muestran clinas latitudinales más fuertes, incrementando en frecuencia con la latitud (Krimbas 1992) y su variación temporal (Orengo y Prevosti 1996) está correlacionada con la temperatura.

1.5. Variación fenotípica

La presencia de variación fenotípica en la naturaleza, que es posteriormente moldeada por la acción de la selección, es el centro de la evolución biológica. Formalmente se dice que la selección es el resultado de tres condiciones necesarias (Lewontin 1970; Cadevall 1988; Soler 2002): la existencia de variación fenotípica entre los individuos de una población; la existencia de diferencias en las capacidades de supervivencia y/o reproducción de los distintos fenotipos, es decir en su aptitud o *fitness*; y la heredabilidad de esas diferencias en la aptitud.

Por otro lado, el fenotipo está definido por la expresión de muchos genes diferentes, y también por el producto de las interacciones del genotipo con el ambiente. Así, dentro de la variabilidad fenotípica se pueden concebir dos componentes: el componente genético o el conjunto particular de genes que posee el individuo y, el componente ambiental que es el conjunto de todas las causas no genéticas que influyen en el valor fenotípico. El genotipo da un cierto valor fenotípico al individuo, pero este valor se ve afectado por el ambiente.

Asimismo, la variación ambiental puede tener dos efectos sobre la variabilidad fenotípica: Los efectos ambientales generales o externos, provocados por factores de influencia (ejemplo, temperatura, salinidad, densidad) que son compartidos por grupos de individuos (variación

dentro de grupos de individuos) y, los efectos ambientales específicos o internos producidos por las desviaciones residuales del fenotipo que podrían ser especificadas por las bases del genotipo (variación entre individuos; ruido de desarrollo, ejemplo mutaciones) y su interacción con los efectos ambientales generales. Tales desviaciones son únicas en los individuos y son ampliamente impredecibles.

La varianza asociada a estos efectos ambientales específicos, puede ser estimada mediante el uso de líneas completamente endogámicas en las que no hay varianza genética. Cuando medimos los valores fenotípicos de un carácter en individuos genéticamente diferentes que han crecido en el mismo ambiente, las diferencias entre unos y otros se deben exclusivamente a causas genéticas. Si no hubiera influencia del genotipo (individuos con el mismo genotipo) todo el valor fenotípico se debería al efecto ambiental externo.

1.5.1. Control de la variación fenotípica

Cuando las condiciones ambientales cambian, los organismos y las poblaciones deben también modificarse a fin de resistir la presión de dichas variaciones, de tal modo que el desarrollo de los individuos puede verse alterado en mayor o menor grado. Así, los individuos pueden presentar una fuerza fenotípica que conduce a fenotipos consistentes u óptimamente adaptados a un ambiente determinado sin presentar mayores cambios en su fenotipo. Los tres procesos siguientes están considerados como mecanismos de control de la variabilidad fenotípica.

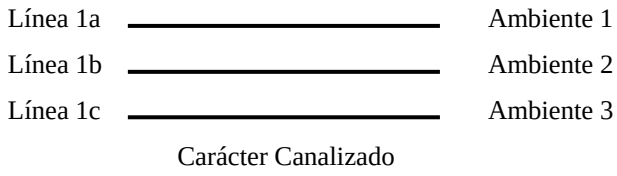
1.5.1.1. Canalización

Se considera una fuerza de restricción morfogenética (Waddigton 1942; Gilbert 2003) por la que el desarrollo parece ser amortiguado, y las ligeras desviaciones del genotipo o ligeras perturbaciones en el ambiente no conllevan necesariamente a la producción de fenotipos anormales. Los genéticos evolutivos la definen como la tendencia de los caracteres o rasgos a desarrollar una reducción en la variabilidad (Gibson y Wagner 2000; Meiklejohn y Hartl 2002). Este proceso permite la producción de un fenotipo específico o básico bajo diferentes condiciones ambientales y genéticas y, por tanto, disminuye la varianza interindividual dentro de grupos, reduciendo la sensibilidad genética a las condiciones ambientales. Por esto, la canalización puede estimarse estudiando la variación interindividual.

Existen dos formas de conducir un estudio de canalización. Dworkin (2005) define la primera como la norma de reacción o propiedad del genoma, y la segunda como una variación aproximada. Ambas definiciones conducen a diferencias métricas en el estudio de

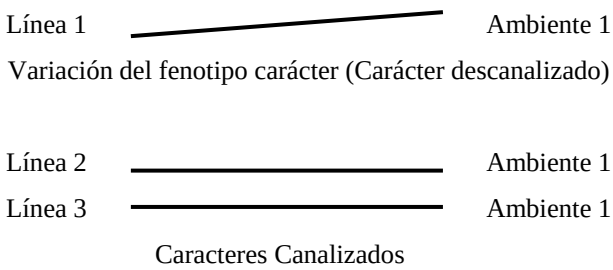
canalización. La primera, la canalización como norma de reacción de la media (RxNM) de un carácter, busca determinar si las líneas de un estudio (genotipos independientes) están canalizadas respecto a diferentes ambientes; considerando una línea canalizada cuando no se observan efectos ambientales en la expresión del carácter, manteniéndose estable en los diferentes ambientes y sus valores no cambian (ver figura 23).

Figura 23: Canalización por norma de reacción



En la variación aproximada de canalización, que es la variación métrica, se busca saber cómo la medida de variación cambia dentro de una línea. Esto permite identificar entre líneas genéticamente distintas en un ambiente común determinado, cuales están mejor canalizadas que otras porque presentan menos variación del carácter dentro de líneas (ver figura 24). En una prueba de canalización se infiere la canalización por la descanalización de un sistema en un ambiente de estrés (genético o exógeno) que causa perturbaciones en la expresión normal del carácter marcador o en estudio y por tanto se observa un incremento significativo en la variación fenotípica entre las líneas en los diferentes ambientes de estrés. Cuando una línea está más canalizada, su media muestra menos cambios a través de los ambientes, con respecto al resto de líneas.

Figura 24: Canalización por variación aproximada



Aunque no hay un diseño específico para los experimentos de canalización, hay unos tratamientos experimentales que dirigen este tipo de estudios:

a. Control de la cantidad de variación genética entre líneas o poblaciones: La canalización es una propiedad del genotipo y como cada individuo tiene un genotipo único, indagar sobre la canalización de una población bajo varios ambientes podría resultar un tanto

difícil por la respuesta diferencial de los diferentes genotipos. Por esto, es importante controlar la variación genética dentro de líneas. En la mayoría de los sistemas genéticamente manipulables, y en las especies que se producen clonalmente, es posible obtener individuos estrechamente relacionados por endogamia o cruces genéticos controlados (como los procedimientos de extracción de cromosomas en *Drosophila*). De esta manera, un mismo genotipo puede ser examinado bajo múltiples ambientes y además, se puede hacer un muestreo razonable del genotipo.

b. Necesidad de un muestreo independiente y múltiple (a través de genotipos y no de individuos): Dado que cada línea representa una simple muestra genética, la medición de múltiples individuos dentro de la línea incrementa esencialmente el muestreo de una medida sencilla, dando una mejor estima. Cuando se usan múltiples líneas es importante considerar la independencia de las líneas (como líneas no genéticamente relacionadas). Otra forma de mejorar la estima es la replicación de los datos, donde el mismo genotipo es remuestreado y las muestras son tratadas como independientes.

c. Control del fondo genético por comparación entre tratamientos: Este punto es indispensable en estudios de canalización genética donde diferentes cromosomas (mutantes u otro marcador) son comparados por sus efectos de canalización. Para sistemas genéticamente manipulables es posible tener genotipos controlados donde un cromosoma (mutante u otro marcador) dado es cruzado en una línea endogámica dentro de muchos fondos genéticos de interés. Esto permitiría la comparación ideal entre el mutante y los individuos del tipo salvaje, los cuales son esencialmente idénticos excepto para el alelo en estudio. Si el fondo genético no se controla, entonces serán confusos los resultados de los efectos observados de los loci (o marcadores) en estudio en el fondo genético de la línea.

1.5.1.2. Estabilidad del desarrollo

Es la habilidad de los organismos para amortiguar el ruido aleatorio que se presenta o surge espontáneamente como consecuencia de la variación estocástica en los procesos celulares que están involucrados en el desarrollo de estructuras morfológicas (Klingenberg 2004). El control genético de la estabilidad de desarrollo esta íntimamente unido a la variación genética no aditiva de los caracteres morfológicos de interés. La dominancia y la epistasis tienen un papel importante en la arquitectura genética de la estabilidad del desarrollo. Los procesos moleculares y celulares que constituyen el desarrollo son inherentemente variables, pero contribuyen al ensamblaje fidedigno del intrincado plan de organización del cuerpo. Los mecanismos que llevan a este nivel de fiabilidad del fenotipo son conocidos como estabilidad

del desarrollo. Sin embargo la naturaleza de estos mecanismos aun no es bien conocida. La estabilidad del desarrollo es un conjunto de una amplia clase de fenómenos de amortiguamiento del desarrollo, los cuales también incluyen canalización contra efectos genéticos y ambientales.

Este grado de resistencia contra las posibles perturbaciones es difícil de medir, por esto es mas fácil cuantificar su contrario, la inestabilidad del desarrollo, que es entendida como la imprecisión que conduce a la variabilidad morfológica incluso cuando la genética y las condiciones ambientales se mantienen constantes. Puede medirse convenientemente como las diferencias aleatorias entre los lados izquierdo y derecho (asimetría bilateral) del organismo o de las partes de un organismo (variación intra individual).

En organismos o partes bilaterales y simétricas, la asimetría fluctuante ofrece un medio fácil para estudiar la inestabilidad de desarrollo. Ambos lados o partes comparten el mismo genoma y usualmente se desarrollan bajo condiciones ambientales casi idénticas y por tanto la variación de asimetría alrededor de su promedio es debido a las fluctuaciones aleatorias de los procesos de desarrollo, y puede ser usado como una medida de la inestabilidad de desarrollo (Klingenberg 2003). La inestabilidad es pues el producto resultante entre los efectos del ruido de desarrollo y la capacidad del sistema para amortiguarlo. De esta manera, la asimetría fluctuante puede aumentar si aumenta el ruido de desarrollo o lo que es lo mismo, si disminuye la estabilidad de desarrollo.

1.5.1.3. Plasticidad

Habilidad de un individuo a expresar un fenotipo bajo una serie de circunstancias ambientales y otro fenotipo bajo otras circunstancias. Los fenotipos expresados pueden ser discontinuos, obteniendo formas discretas (poli-fenotípicas) o pueden ser intervalos continuos de fenotipos potenciales. Son alternativas al cambio genético que permite a las poblaciones adaptarse a las condiciones cambiantes del medio ambiente, incrementa la variabilidad entre grupos de individuos y produce diferentes fenotipos en diferentes entornos.

Estudiando líneas independientes en las cuales se han muestreado múltiples individuos para un valor fenotípico en ambientes diferentes, y para los que se asume una varianza ambiental (interna) similar, la plasticidad y la canalización se muestran como características opuestas del mismo fenómeno (Nijhout y Davidoowitz 2003). Así, la plasticidad del fenotipo se manifiesta entonces, en un cambio de la media del carácter a través de los diferentes ambientes.

1.5.2. Análisis de asimetría

Las estructuras de los organismos de simetría bilateral, ofrecen una forma ideal de normalidad. Las desviaciones de la simetría bilateral se consideran como un índice de homeostasis y pueden utilizarse como indicador de las fuerzas fenotípicas actuantes en los individuos. Estas desviaciones de la normal son comúnmente descritas por distribuciones de frecuencia para la diferencia entre los lados [derecha – izquierda (R-L)]. Cada distribución de frecuencia exhibe usualmente uno de los tres patrones (ver figura 25): asimetría fluctuante, asimetría direccional y antisimetría. Cada uno de estos patrones indica la variación de un carácter en particular (entre un lado y el otro) exhibida por una muestra de individuos, como respuesta durante su ontogenia al estrés genético (intrínseco) o ambiental (extrínseco). Así, el grado de asimetría entre un carácter par (ejemplo las dos alas de la mosca) sería reflejo de la estabilidad durante el desarrollo del organismo. El parámetro más utilizado para medir esa estabilidad es la asimetría fluctuante (FA) (Pither y Taylor 2000; Palmer y Strobeck 2003).

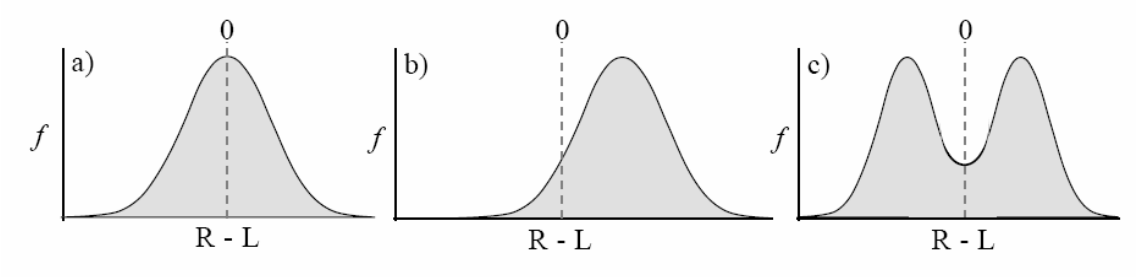


Figura 25: Distribuciones mas comunes de R-L en organismos de simetría bilateral: a) asimetría fluctuante (FA) b) Asimetría direccional (DA) y c) antisimetría (*Platykurtic* o bimodal).

La FA es un patrón de variación bilateral de una muestra de individuos donde la media de R-L es cero y la variación está distribuida normalmente alrededor de esta media (figura 25a). Este patrón de variación bilateral puede aumentar por muchos procesos del desarrollo, entre estos, los relacionados con los presumiblemente independientes pero contrarios procesos de la inestabilidad del desarrollo (o ruido de desarrollo) y los procesos de la estabilidad del desarrollo. Los primeros tienden a un desarrollo anormal, que pueden ser de carácter intrínseco del individuo, por ejemplo, pequeñas diferencias aleatorias en las tasas de división celular, cambios en el crecimiento y la forma celular, efectos de ruido termal en procesos enzimáticos o pequeñas diferencias aleatorias en las tasas de procesos fisiológicos entre las células. Los procesos de estabilidad del desarrollo tienden a un desarrollo normal, resistiendo o amortiguando los trastornos del desarrollo, por ejemplo, sistemas de retroalimentación o

reacción negativa que regulan la actividad enzimática (tasas de concentración y de catálisis) dentro y entre células, regulación nerviosa central de estructuras no contiguas o regulación hormonal de estructuras no contiguas. De esta manera, las diferencias en la FA entre muestras pueden aumentar por la diferencia en cualquiera de estos dos fenómenos, indicando su contribución relativa y la homeostasis general o el flujo de estabilidad de desarrollo.
La FA se considera por tanto, como un índice de homeostasis y puede utilizarse como indicador del estrés genético (consanguinidad, homocigosis, hibridación, mutación, selección) y ambiental (temperatura, alimentación, polución). Se puede utilizar para la comparación entre poblaciones o individuos dentro de una misma población. A nivel de una población, los individuos pueden diferir en el grado de asimetría y esta diferencia puede deberse en parte a diferencias genéticas.

Por otro lado, las desviaciones de simetría aparecen correlacionadas con diferencias en aptitud, particularmente en las características que afectan directamente el rendimiento de los individuos (Thornhill 1991; Moller 1994). Una baja aptitud está asociada con una alta FA (Biemont 1983; Clarke y McKenzie 1987; Quattro y Vrijenhoek 1989). Así como también, individuos con baja FA muestran una mayor fecundidad, tasas de crecimiento elevadas y mayor supervivencia (Mitton y Grant 1984; Palmer y Strobeck 1986; Parsons 1992; Moller 1997; Moller y Swaddle 1997). Por tanto, el estudio de los caracteres bilaterales brinda un método apropiado para medir las variaciones del fenotipo, variación entre individuos y dentro de individuos, en el estudio de los factores que pueden influir en la respuesta de los individuos con un genotipo dado en un ambiente determinado.

La asimetría direccional (DA, ver figura 25b) así como la antisimetría (AA, ver figura 25c) son consideradas variantes pero no son indicadores del estrés, aunque si pueden influir en el valor de la FA, por eso es importante determinar su presencia en un análisis de la variación fenotípica. La DA puede tener una base genética significativa (Palmer & Strobeck 1986; Palmer 1994) y por lo tanto, no apta como una medida de la inestabilidad del desarrollo (Lens & Van Dongen 2000).

En la antisimetría, el patrón de variación bilateral en una muestra de individuos tiene una diferencia estadísticamente significativa entre lados, pero el lado que es más grande varía entre los individuos al azar (figura 25c), detectada por la prueba estadística para desviación de distribuciones de frecuencias (R-L) de normalidad en la dirección de platicurtosis o distribución bimodal (pico menos extenso <3) o valores negativos de curtosis; media

normalmente cero de R-L. Y en la asimetría direccional, el patrón de variación bilateral de la muestra de individuos tiene una diferencia estadísticamente significativa entre lados, pero el lado que es más grande es generalmente el mismo (figura 25b), mostrando una dirección constante, y su distribución en la población muestra una aparente desviación hacia un lado en particular (Moller y Swaddell 1997; Palmer 1994; Polak 2003), detectado por la prueba estadística para desviaciones de la media R-L de cero.

OBJETIVOS

1.5. Objetivo General

Estudiar los cambios en el polimorfismo para inversiones cromosómicas y la morfometría del ala en una población experimental de *Drosophila subobscura* sometida a diferentes regímenes térmicos en un periodo de 2 años para comprobar el posible efecto de la temperatura sobre dichos caracteres.

Para el logro de este objetivo general se establecieron los siguientes objetivos específicos:

1.5.2. Objetivos Específicos

- Seleccionar un número representativo de individuos de una población americana para fundar la población experimental que estará bajo régimen de temperaturas diferentes (13ºC, 18ºC y 22ºC).

- Mantener dicha población experimental en condiciones de laboratorio bajo control exhaustivo de las condiciones experimentales (densidad de población y temperatura).

- Estudiar las frecuencias de los ordenamientos cromosómicos en cada una de las poblaciones en un periodo de 2 años para identificar los cambios posibles que estén relacionados con la temperatura.

- Estudiar la morfometría del ala para identificar los cambios posibles de tamaño y forma relacionados igualmente con la temperatura, mediante el uso de las medidas lineales de 13 *landmarks* en las intersecciones de las venas del ala para el cálculo de longitud promedio del ala (WL) y el tamaño del centroide (CS) en el análisis de tamaño y, los análisis *Procrustes* en el estudio de la forma del ala en las poblaciones experimentales.

- Fundar líneas isocromosómicas genéticamente homogéneas de los ordenamientos cromosómicos del cromosoma O de *D. subobscura* y mediante cruces recíprocos entre éstas, criadas bajo dos temperaturas de desarrollo (18ºC y 23ºC), ver los efectos del cariotipo, homocigosis y la temperatura en la forma del ala y la asimetría bilateral.

- Analizar los efectos de la variación genética clinal (polimorfismo de inversión) en la dimensión total del ala (tamaño y forma) y la simetría bilateral, mediante el uso de métodos aplicados de morfometría geométrica en el contexto de la genética cuantitativa, considerando las consecuencias de la endogamia y la temperatura en los componentes de la homeostasis del desarrollo, la canalización y la estabilidad del desarrollo (DS), y la relación entre estos.

2. ARTICULOS

2.1. Artículo 1

Quantitative – genetic analysis of wing form and bilateral asymmetry in isochromosomal lines of Drosophila subobscura using Procrustes methods.

Autores: Pedro F. Iriarte, **Walkiria Céspedes** y Mauro Santos,

Revista: Journal of Genetics (*special issue on Evolutionary Genetics: the* Drosophila *Model),* 82: 95 – 113. 2003.

2.2. Artículo 2

Swift laboratory thermal evolution of wing shape (but not size) in Drosophila subobscura and its relationship with chromosomal inversion polymorphism.

Autores: Mauro Santos, Pedro F Iriarte, **Walkiria Céspedes**, Joan Balanyà, Antonio Fontdevila y Luís Serra.

Revista: Journal Evolutionary Biology, 17: 841 – 855. 2004.

2.3. Artículo 3

Temperature – related genetic changes in laboratory populations of Drosophila subobscura: evidence against simple climatic – based explanations for latitudinal clines and correlated continent wide shifts.

Autores: Mauro Santos, **Walkiria Céspedes**, Joan Balanyà, Vincenzo Trotta, Federico Caboly, Antonio Fontdevila y Luís Serra.

Revista: The American Naturalist, 165: 258 – 273. 2005.

2.4. Artículo 4

Genetics and geometry of canalization and developmental stability in Drosophila subobscura.

Autores: Mauro Santos, Pedro F. Iriarte y **Walkiria Céspedes**

Revista: BMC Evolutionary Biology, 5: 7. 2005

Quantitative-genetic analysis of wing form and bilateral asymmetry in isochromosomal lines of *Drosophila subobscura* using Procrustes methods

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Abstract

Fluctuating asymmetry (FA) is often used as a measure of underlying developmental instability (DI), motivated by the idea that morphological variance is maladaptive. Whether or not DI has evolutionary potential is a highly disputed topic, marred by methodological problems and fuzzy prejudices. We report here some results from an ongoing study of the effects of karyotype, homozygosity and temperature on wing form and bilateral asymmetry using isochromosomal lines of *Drosophila subobscura*. Our approach uses the recently developed methodologies in geometric morphometrics to analyse shape configurations of landmarks within the standard statistical framework employed in studies of bilateral asymmetries, and we have extended these methods to partition the individual variation and the variation in asymmetries into genetic and environmental causal components. The analyses revealed temperature-dependent expression of genetic variation for wing size and wing shape, directional asymmetry (DA) of wing size, increased asymmetries at suboptimal temperature, and a transition from FA to DA in males as a result of increase in the rearing temperature. No genetic variation was generally detected for FA in our samples, but these are preliminary results because no crosses between lines were carried out and, therefore, the contribution of dominance was not taken into account. In addition, only a subset of the standing genetic variation was represented in the experiments.

[Iriarte P. F., Céspedes W. and Santos M. 2003 Quantitative-genetic analysis of wing form and bilateral asymmetry in isochromosomal lines of *Drosophila subobscura* using Procrustes methods. *J. Genet.* **82**, 95–113]

Introduction

Developmental instability (DI) is the result of processes that disturb development along a developmental trajectory within a particular environment (Palmer 1994; Nijhout and Davidowitz 2003). A measure of within-individual variation, which is the finest level at which phenotypic variance can be reckoned (Lynch and Walsh 1998, pp. 112–116), provides a quantifiable expression of underlying DI that is referred to as fluctuating asymmetry (FA, i.e. small random deviations from symmetry in otherwise bilaterally symmetrical characters; Van Valen 1962; Palmer and Strobeck 1986; Leary and Allendorf 1989; Markow 1995; but see Palmer and Strobeck 2003, p. 281, for a cautionary remark on the relationship between FA and DI). Insofar as both sides of a symmetrical structure are uniformly controlled by the same genes (barring unusual somatic mutation or somatic recombination), the subtle nondirectional differences typically found between right and left sides of bilateral traits are expected to arise owing to inherent nongenetic developmental noise (e.g. Waddington 1957; Palmer and Strobeck 1986; Palmer *et al.* 1993). Mather (1953) was the first to suggest, however, that FA had a genetic basis. More recently, interest in FA as a putative reliable cue of the overall genetic quality of an individual has led some authors to claim that FA (and hence DI) does indeed have a positive heritability and is

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Keywords. directional asymmetry; *Drosophila subobscura*; fluctuating asymmetry; geometric morphometrics; isochromosomal lines; Procrustes analysis; wing shape; wing size.

therefore biologically and evolutionary relevant on its own (reviewed in Møller and Swaddle 1997). These claims have raised strong (sometimes acid) controversies and have generated quite a number of meta-analyses on published estimates of FA heritabilities. Though one positive outcome of this debate has been more rigorous and focussed research (Polack 2003), the topic of the genetical basis of FA still remains completely unresolved (Santos 2002; Fuller and Houle 2003).

In a recent quantitative-genetic study of wing size asymmetry, one of us found low (~ 0.04) but significant heritabilities of FA in wing length and width in *Drosophila buzzatii* (Santos 2002). Some findings were, however, difficult to reconcile with those positive heritabilities, besides the fact that the additive genetic correlation for size between sides was not significantly different from +1. If variation that is due to stochasticity in development is the only real cause of FA (see Nijhout and Davidowitz 2003), there are at least three possible explanations for those results. First, they might have simply been false positives. However, the detection of similarly low (but nonsignificant) wing size FA heritability in two previous experiments (Santos 2001) casts some doubts on this explanation. Second, the random noise component is independent of genotype, but genetic variance for trait size alone could give rise to low heritabilities of FA (Klingenberg and Nijhout 1999). Although wing size in *Drosophila* usually shows intermediate heritabilities, a problem with this explanation is that there is no relationship between trait size and FA in *D. buzzatii* (Santos 2001, 2002; see also Loeschcke *et al*. 1999, 2000). Third, the sensitivity to developmental noise in a random-mating population may be genotype dependent. This alternative arises from developmental models where there is a nonlinear mapping between development and FA (see Klingenberg 2003a), and we find it particularly attractive and thought-provoking.

Stochastic mechanisms in gene expression can explain the substantial phenotypic variation usually observed in clonal populations (Elowitz *et al*. 2002). Alternatively, for bilaterally symmetrical structures controlled by the same genes, this inherent stochasticity would lower the between-side correlation in gene expression, likely resulting in FA. The important point for FA to display positive heritability is, however, whether or not the noise is genotype dependent. Models of stochastic gene expression predict that intrinsic noise should increase as the amount of transcript decreases (Rao *et al*. 2002; Swain *et al*. 2002), and recent evidence shows that the level of gene expression in eukaryotic cells is indeed strongly influenced by transcription (Blake *et al*. 2003). Although at present it is largely unknown how widespread the genetic variation in transcript levels is, recent studies in humans have identified genes whose transcript levels differed greatly among unrelated individuals (Cheung *et al*. 2003). In addition, it has already been shown that noise has a genetic component; *recA* mutants in *E. coli* are twice as noisy as their wild-type counterparts (Elowitz *et al*. 2002). In summary, if the amount of noisiness is genetically determined, then different genotypes might exhibit different levels of DI. In this case, a reliable measure of genotype-dependent FA would clearly depend on replication, both at the genotype and at the experimental levels.

A basic problem in using FA as an estimate of an individual's ability to buffer its phenotype from genetic or environmental perturbations or both is the low signal/noise ratio. FA correlates only loosely with underlying DI because it is an attempt to estimate a variance with two data points (right and left; see Whitlock 1996, 1998; Van Dongen 1998). However, if the standard model of the relationship between FA and DI (Palmer and Strobeck 1992; Houle 1997, 2000; Leung and Forbes 1997; Gangestad and Thornhill 1999) provides a convenient approximation to reality, the putative genetical basis of FA could be detected by replicating genotypes to increase the signal. For some *Drosophila* species this can be attained from a classical and widely used technique that allows rendering individuals homozygous for virtually all genes carried by an entire chromosome, thus obtaining isogenic or isochromosomal lines (see, for example, Wallace 1981). Additional and reliable information about the relationships among genetic or environmental stresses or both with FA levels could also be gained by using the established isogenic lines (see also Woolf and Markow 2003).

An equally important problem in studies of FA is the choice of traits. For metric traits it is generally assumed that each trait value reflects an independent sample from a normal distribution with mean equal to its expected phenotypic value and variance reflecting the amount of random noise and the degree of DI (see, for example, Van Dongen *et al*. 2003). If this is the case, the presence of between-trait correlations in the unsigned FA [abs (left–right)] could be indicative of an organism-wide DI (Møller and Swaddle 1998), but the problem is the low correlation between single-trait FA and the presumed underlying DI (see above). On the other hand, if there is a developmental connection between the traits studied a correlation between the signed asymmetries of traits is expected (see Van Dongen *et al*. 1999; Santos 2002; Klingenberg 2003b), which would hamper the interpretation of between-trait correlations in the unsigned FA, thus rendering those traits unsuitable for studying organism-wide DI. In contrast, relevant knowledge on developmental interactions and morphological integration can now be gained by comparing the patterns of covariation among asymmetries of traits (Klingenberg *et al*. 1998; Klingenberg and McIntyre 1998; Debat *et al*. 2000; Klingenberg and Zaklan 2000). As pointed out by Klingenberg (2003b), this approach departs from more traditional research on FA but opens a new direction of research at the interface between evolutionary and developmental biology.

We report here some initial results from an ongoing study of the effects of karyotype, homozygosity and temperature on wing form and bilateral asymmetry in isochromosomal lines of *Drosophila subobscura*. This is a particularly inversion-rich species, with up to 38 natural chromosomal arrangements already reported for the largest chromosome O (Krimbas and Loukas 1980), for which a balanced marker strain has been developed (Sperlich *et al.* 1977). A large number of isochromosomal lines in an otherwise homogeneous genetic background were obtained from a stock collected at Puerto Montt (Chile), which is a New World colonizing population that harboured a total of seven chromosome O arrangements (including the recombination-derived and sporadically found O_7 arrangement; see Balanyà *et al.* 2003; Santos *et al.* 2004). Here we have used a subset of lines that belong to the O_{st} class, and have studied wing size and shape using the framework of geometric morphometrics (Bookstein 1991; Dryden and Mardia 1998). We show that the genetic variability for overall wing shape was comparable to that for wing size, although results were temperature dependent. On the other hand, directional asymmetry (DA) has been detected for wing size but not shape, and no genetic variation was observed for FA. However, it should be borne in mind that these are preliminary results because (i) no crosses between lines were carried out and, therefore, the contribution of dominance was not taken into account; and (ii) most genetic variation in New World colonizing populations of *D. subobscura* is likely apportioned among chromosome arrangements.

Materials and methods

Base stocks and fly handling

The *D. subobscura* base population originated from 93 isofemale strains derived from a large outbred stock collected by Drs J. Balanyà, G. W. Gilchrist, R. B. Huey and M. Pascual at Puerto Montt (Chile; 41°28′S) in November 1999. The isofemale lines were kept at 18°C for more than one year (~16 generations) prior to the establishment of a set of three replicated thermal selection stocks (see Santos *et al.* 2004). In November 2001, eggs were collected over three consecutive days from the populations kept at the experimental temperature of 18°C, and were placed in 130-ml bottles (~200 eggs per bottle) containing 50 ml of David's killed-yeast *Drosophila* medium (David 1962). A random sample of 300 males (100 males per replicate) from those that emerged from the bottles were used to obtain isochromosomal lines.

***Extraction of O chromosomes*:** The procedure used to obtain isochromosomal lines for the O chromosome (homologous to arm 3R in *D. melanogaster*; Powell 1997, p. 307) in an otherwise homogeneous genetic background is schematically shown in figure 1. Briefly, wild-type males were individually crossed to three or four virgin females from the *ch-cu* marker strain, which is homozygous for the morphological recessive markers on the O chromosome *cherry* eyes (*ch*) and *curled* wings (*cu*) (Koske and Maynard Smith 1954). The genetic background of the *ch-cu* strain is highly homogeneous and fixed for the so-called standard gene arrangements in all major acrocentric chromosomes but chromosome O, where it is fixed for gene arrangement O_{3+4} (Lankinen and Pinsker 1977). A single F_1 progeny of each cross was backcrossed to *ch-cu* females and the scheme was repeated for five generations. After four generations of crosses the lines were identified for chromosome arrangements at all chromosomes by microscope inspection of up to five third-instar larva salivary gland squashes to look for inversion loops in polytene chromosomes. This allowed identification of the isolated O gene arrangements and to check for presence of undesirable nonstandard chromosomal arrangements on the

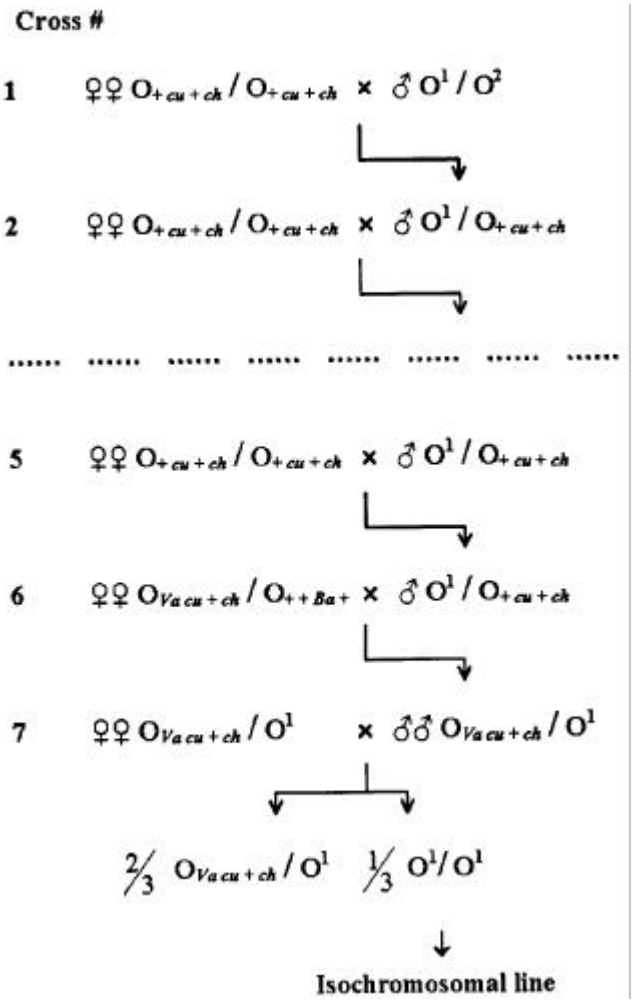

Figure 1. Mating scheme used to establish the isochromosomal lines in an otherwise homogeneous *ch-cu* genetic background.

other three major autosomes (named E, J and U; see Krimbas and Loukas 1980) carried by the founding wild-type males. To obtain the isochromosomal strains a single male from the selected lines was crossed to two virgin females from the *Va*/*Ba* balanced marker stock (Sperlich *et al*. 1977). This stock carries the dominant lethal genes *Varicose* (*Va*) and *Bare* (*Ba*) on the O chromosome and was derived from the *ch-cu* strain. Because expression of the *Ba* gene is highly variable and affected by modifiers located on the O chromosome (Alvarez *et al*. 1981), we relied only on the *Va* marker to obtain the isochromosomal lines. A total of 114 lines were obtained; their viability distributions are shown in figure 2. Viabilities were estimated after pooling the progeny raised from two replicated vials; each vial was set up with three $O_{Va\ cu+ch}/O^1$ females crossed to two $O_{Va\ cu+ch}/O^1$ males (cross # 7 in figure 1). Twelve days after crosses were made all parents were discarded. The offspring counts were continued until all flies emerged from the vials, with a total of 25,425 flies (harmonic mean = 185.7 flies per line). Viability was expressed as the ratio

$$\frac{\text{number of wild-type flies}}{\text{total number of flies}}.$$

The viability distribution for all lines (figure 2a) had the usual pattern in this kind of experiments (see, for example, Wallace 1981, p. 67), with 18 lines (15.8%) having a viability lower than 0.1. The number of lines for each chromosomal class (figure 2b) was proportional to the frequencies of chromosome O arrangements in the founding thermal stocks (Santos *et al*. 2004). As expected, those lines carrying the arrangement O_5 were lethal (figure 2b; see Mestres *et al*. 1992).

The lines used in the experiments were genotyped for microsatellite loci on the chromosome O and found to be homozygous, checked again for all chromosome gene arrangements after individually crossing the isogenic males to three or four *ch-cu* virgin females, and had a quasinormal viability (defined as >0.25). All crosses were made at 18ºC (12 h : 12 h light : dark cycle), and fly handling was done at room temperature using CO_2 anaesthesia on flies not less than 6 h after eclosion.

***Experimental design*:** The data used here are from an ongoing study of the effects of karyotype, homozygosity and temperature on wing asymmetry in *D. subobscura*. We report results from isochromosomal lines that belong to the O_{st} class. Isochromosomal lines were kept in 130-ml bottles with low adult density to standardize the conditions of rearing before egg collections. Eggs were collected from each of 10 O_{st} lines from spoons containing nonnutritive agar with a generous smear of live yeast, and placed in three 2 cm × 8 cm vials with 6 ml of food (26 eggs per vial) at 18ºC on the same incubator shelf. Similarly, eggs from a subset of five isochromosomal lines were placed in vials at 23ºC (suboptimal temperature). Emerging flies (no less than two or three days old) were stored in Eppendorf tubes with a 3 : 1 mixture of alcohol and glycerol at 4°C before wing measurements.

Wing size and shape

***Definitions*:** Morphometrics involves quantitative study of form, and it is naturally understood that form consists of size and shape (Needham 1950). An important contribution of geometric morphometrics is the clear definition of size and shape (Dryden and Mardia 1998). Size is defined as any positive real-valued function from a landmark configuration (i.e. a set of points that can be precisely located) matrix X that satisfies the condition $g[aX] = ag[X]$ for any positive scalar a. The shape of a set of p landmark points is the geometrical information of the configuration of points that is invariant to translation (variation in the position of the configuration in the digital image), rotation (variation in the orientation of the configuration), and rescaling (variation in size).

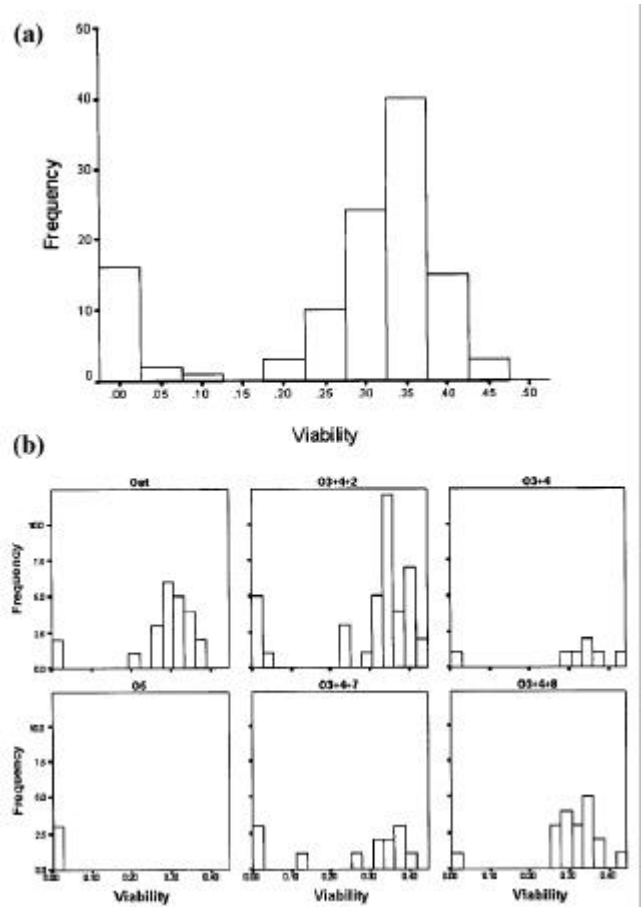

Figure 2. Viability distributions for the isochromosomal O lines: (a) all 114 lines; (b) grouped according to chromosomal arrangement.

***Wing measurements*:** Wings from both sexes were removed and fixed in DPX under coverslips on microscope slides. The data consisted of *x* and *y* coordinates of 13 morphological landmarks (figure 3). All landmarks used are at the intersections of wing veins or at points where veins reach the wing margin and are easy to locate precisely, and can therefore be considered type 1 landmarks according to Bookstein (1991, pp. 63–67) or anatomical landmarks according to Dryden and Mardia (1998, p. 3). Wings were digitized by one of us (P. F. I.) using a compound microscope (Zeiss Axioskop) with low-power objective (2.5×) and attached video camera (Sony CCD-Iris) connected to a PC with MGI VideoWave software. Calibration of the optical system was checked in each session. All wings were digitized and measured two times in different sessions as follows. Both the left and right wings were digitized during a given session, and after an entire round on all individuals, the same process was repeated again. The *x* and *y* coordinates of the morphological landmarks were recorded for each wing in a similar fashion using the image processing and analysis program Scion Image (based on the NIH-Image for Macintosh and available at http://www.scioncorp.com). The procedure we used guaranteed that the observer was blind with respect to the results from previous measurements.

Statistical analyses of wing size and shape, and asymmetry

Overall wing size was analysed here using centroid size (defined as the square root of the sum of squared distances of a set of landmarks from their centroid or, equivalently, the square root of the sum of the variances of the landmarks about that centroid in *x* and *y* directions; Slice *et al*. 1996) as the dependent variable (referred to as CS). Wing shape (WS) was characterized by the geometrical configuration of the morphological landmarks after variation in wing size was removed.

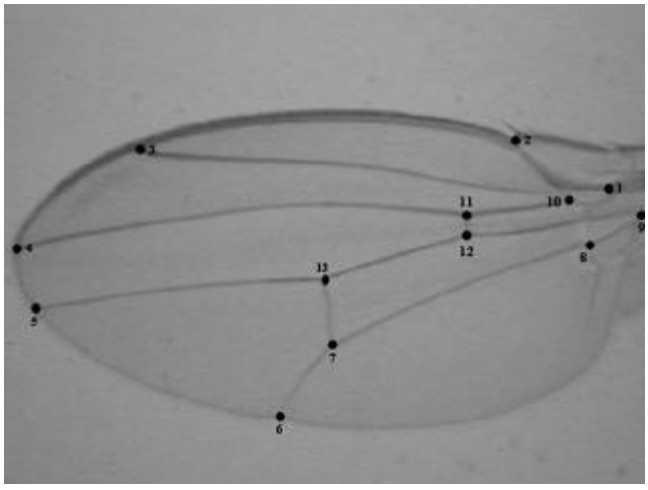

Figure 3. Image of *Drosophila subobscura* left wing indicating the thirteen landmarks used in this work.

To assess asymmetry we used the conventional mixed-model, two-way analysis of variance where individuals is a random effect and sides is a fixed effect (Palmer and Strobeck 1986; Palmer 1994). In this ANOVA the main effect of individual flies stands for phenotypic variation in the trait (CS or WS), the main effect of body sides is for directional asymmetry (DA) and tests whether or not the signed differences between the left and right wings [designated as $(L - R)$] have a mean of zero, the interaction term is a measure of fluctuating asymmetry (the variation in left–right differences among individuals) provided that there is no genetic variation for DA (see Santos 2002), and the error term provides an estimate of the measurement error. In addition, if individuals can be grouped into families or lines (as happens here), the variation among individuals can be further decomposed to test for genetic effects on the trait; and the variation due to the interaction term can also be decomposed to test for genetic effects on DA (Santos 2001, 2002). Following Sokal and Rohlf (1981, p. 337) and Leamy (1999), mean squares for individuals were tested over the error mean squares whereas mean squares for sides were tested over the individuals × sides interactions.

To test for size dependence of FA, regressions of unsigned $|L - R|$ asymmetries on centroid sizes were performed and in no case were the slopes statistically different from zero (females 18ºC: $b = -0.0211$, $F_{1,98} = 2.94$, n.s.; males 18ºC: $b = 0.0144$, $F_{1,98} = 0.47$, n.s.; females 23ºC: $b = 0.0305$, $F_{1,28} = 0.37$, n.s.; males 23ºC: $b = 0.0129$, $F_{1,28} = 0.12$, n.s.).

***Generalized Procrustes analysis for quantifying wing shape asymmetry*:** Procrustes methods allow comparison of configurations of landmarks by optimally superimposing (according to a least-squares criterion) homologous landmarks in two or more specimens to achieve an overall best fit (Rohlf 1990, 1999; Rohlf and Slice 1990; Klingenberg and McIntyre 1998). When several objects (e.g. wings) are fitted using Procrustes superimposition (as was done in the present work) the method has been called 'generalized Procrustes analysis' (GPA; see Dryden and Mardia 1998, pp. 44–47). In studies of 'matching asymmetry' (Mardia *et al*. 2000; Klingenberg *et al*. 2002), where a structure is present in the two sides of a bilateral organism, the landmark configuration of one body side has to be reflected to its mirror image before aligning the corresponding landmarks of both sides. For this purpose, we changed the sign of the *x* coordinate of every landmark for configurations from the right side, thus following our previous convenience of defining asymmetry as the left–right differences among individuals.

After reflections, our analysis used a single Procrustes superimposition to align simultaneously all the landmark configurations within each sex and isochromosomal line. For each sex we used as the 'base configuration' or refer-

ence specimen the Procrustes grand mean shape of the left wing of the species, obtained from 900 females and 1068 males derived from our thermal stocks (see Santos *et al*. 2003). This allows contrasting wing shapes between different lines or crosses. A single overall consensus configuration was computed for each isochromosomal line as the mean coordinates of corresponding landmarks in the aligned configurations (Rohlf and Slice 1990). The final iteration to minimize the sum of the squared distances between the landmarks of all objects in the sample was done without additional scaling and, consequently, we performed a partial Procrustes fit according to Dryden and Mardia (1998; see also Rohlf 1999). (Rescaling the coordinates of each configuration by the scaling option, $1/\cos(r)$ (see Rohlf 1999) would make very little difference, in the order of ~0.001% of the shape variation in the data sets.) The variation in the landmark coordinates that remains after Procrustes superimposition is a complete and nonredundant description of the variation in shape, and the usual linear multivariate methods focus on these coordinates (see below). The details of computation are clearly described in Klingenberg and McIntyre (1998), Klingenberg and Zaklan (2000) and Klingenberg *et al*. (2002).

In this work we used MATLAB (V.6; The MathWorks, Inc. 2002) for morphometric analyses. Some helpful functions in morphometrics from the MATLAB toolboxes Res5 and Res6 developed by R. E. Strauss were also used (available at http://www.biol.ttu.edu/Strauss/Matlab/matlab.htm).

***Procrustes ANOVA: extensions to analyse genetic and environmental components of shape asymmetries*:** As pointed out by Klingenberg and McIntyre (1998) calculation of Procrustes coordinates is based on the algebra of sums of squares, and the variance in the set of optimally aligned landmark configurations can be partitioned in a way analogous to the deviations from a grand mean in conventional ANOVA (Goodall 1991). The coordinates of the Procrustes-aligned configurations (all two replicates of each wing) are therefore amenable to the preceding two-factor mixed-model ANOVA.

The sums of squares for the individuals and sides main effects, individuals × sides interaction effects, and error terms are obtained after performing separated two-factor ANOVAs for each x and y coordinates of the aligned configurations and summing across all corresponding sums of squares. (Alternatively, we can perform a principal component analysis (PCA; see, for example, Jolliffe 1986) to obtain the matrix of scores from the first 22 PCs (see below) and return these scores into the space of the coordinates of the landmarks of superimposed configurations.) To decompose the resulting sums of squares in the among-isochromosomal-lines and within-isochromosomal-lines components, we proceeded in a similar way but used lines instead of individuals as the main effect. The among-lines sums of squares for the individuals main effect are the same as those obtained after performing one-way ANOVAs to the individuals means for each landmark times the number of measurements per individual (2 wings × 2 measurements per wing; see Santos 2001). Similarly, the among-lines sums of squares for the individuals × sides interaction terms can also be obtained from one-way ANOVAs performed on signed $(L - R)$ asymmetries. In this case, L and R are estimated for each x and y coordinates of the aligned configurations as the averages of the left and right wing measurements, respectively. Lack of statistical significance among line means would indicate that there is no genetic variation for DA.

To test for presence of genetic components in wing shape FA we performed one-way ANOVAs on the unsigned $|L - R|$ estimates for each x and y coordinates of the aligned configurations (index FA1 in Palmer 1994) using lines as the main effect and summing across all coordinates to obtain the among-lines and within-lines sums of squares. There are more degrees of freedom in Procrustes ANOVA than in conventional ANOVA (Goodall 1991) because the squared deviations are summed over all the landmark coordinates. Therefore the number of degrees of freedom is that for ordinary ANOVA times the shape dimension; i.e. $2p–4$ for two-dimensional-coordinate data, where p is the number of landmarks.

Since variation around landmarks in Procrustes-superimposed biological configurations does not generally follow the isotropic model (which presumes that there is an equal amount of nondirectional variation at each landmark; see Goodall 1991; Rohlf 2000), degrees of freedom in Procrustes ANOVAs are not independent. Therefore statistical significance of ANOVA effects was tested from permutation tests (another approach would be to use conventional MANOVA; see Klingenberg *et al*. 2002). For the two-way mixed-model ANOVA randomization is a three-stage process (Good 1994; Edgington 1995): (i) random permutations within sides among individuals for the among-individuals F statistics; (ii) random permutations between sides within individuals for the between-sides F statistics; and (iii) random permutation across individuals and sides after subtracting the deviations due to both individuals and sides, and adding the grand mean (see Sokal and Rohlf 1981, p. 330). Each test used 10,000 random permutations.

***Localized variation*:** We followed Klingenberg and McIntyre (1998) and decomposed the Procrustes mean squares for each effect in the two-way ANOVAs according to the landmarks to assess how much of shape variation was due to each landmark. Thus we summed x and y mean squares of each landmark separately and computed the variance components according to the expected mean squares (Sokal and Rohlf 1981). Because the least-square

algorithm tends to spread variation from variable landmarks to the others, this approach should be taken cautiously if one or a few landmarks are much more variable than the rest (Chapman 1990; Walker 2000).

***Shape variability*:** We used PCA to investigate patterns of covariation in the positions of landmarks, which is a usual method in the context of shape analysis (Dryden and Mardia 1998; Klingenberg and McIntyre 1998; Klingenberg and Zaklan 2000). The analyses must use covariance matrices of the coordinates of superimposed landmarks to avoid problems related to rotations of the coordinate system, and principal components coefficients can be presented graphically by drawing lines centred at the mean location of each landmark and ending at an arbitrary number of standard deviations away from that mean in the direction to which the landmark would shift.

To test for the congruence of landmarks displacements between effects (line, FA and digitizing error), both within and between samples, we followed Klingenberg and McIntyre (1998; see also Debat *et al*. 2000). Thus, within each sample we first computed matrices of sums of squares and cross-products (SSCP) for individuals (both sides and all replicated measurements averaged for each fly), for lines (averages of sides, measurements and flies within lines), for FA (fly × side interaction) from individual left–right differences (wing averages from all measurements), and for measurement error (residual variation of the replicated measurements about the wing average). After dividing the SSCP matrices by the appropriate degrees of freedom, we separated effects according to the expected mean squares by subtracting the within-line variance–covariance (VCV) matrix from the line VCV, and the measurement VCV from the fly × side VCV. For each effect, a PCA of the VCV matrix was performed, and the correlation between PCs was obtained by angular comparisons of component vectors. The statistical significance was assessed by comparing the observed values with a null distribution of absolute angles between 100,000 pairs of random vectors obtained as random points on a 22-dimensional unit sphere (see Klingenberg and Zimmermann 1992). The 0.1% quantile of the distribution of these angles was 50.3º.

***Allometry*:** To test for size effects on shape variation we carried out multivariate regressions of Procrustes coordinates on centroid size (Dryden and Mardia 1998). These regressions generally accounted for less than 3% of total Procrustes sums of squares, and multivariate analyses using the residuals of a regression on centroid size produced results that were qualitatively identical to those of the complete variation. Therefore no size corrections were necessary and we only report the results of analyses of the total shape variation.

Temperature effects on wing size and shape

For those isochromosomal lines that were common to both experimental temperatures three-way mixed-model ANOVAs were performed with effects of temperature and sex assumed to be fixed, and line assumed to be random (for each fly we thus averaged throughout measurements and sides). Following Sokal and Rohlf (1981, p. 383), mean squares for temperature were tested over the T × L interaction, mean squares for sex over the S × L interaction, mean squares for line over the error term, the two-way interaction involving the fixed effects over the three-way interaction, and the two-way interactions involving the random and fixed effects over the error term. The sums of squares for the three-way Procrustes ANOVA were obtained after performing separated ANOVAs for each *x* and *y* coordinates of the aligned configurations and summing across all corresponding sums of squares. In this case statistical significance of ANOVA effects was tested after 10,000 permutation tests (see above).

The computer programs used for statistical data analyses were MATLAB (V.6; The MathWorks, Inc. 2002) together with the collection of tools supplied by the Statistics Toolbox (V.3; The MathWorks, Inc. 2000), and some results were checked with the statistical software packages STATISTICA V.6 (StatSoft, Inc. 2003) and SPSS V.11 (SPSS, Inc. 2001). They were run on a Pentium 4 (1.60 GHz) PC.

Results

Causal components of variation and asymmetry in wing size

The mean values of the average centroid sizes for the two repeated measures on each side are plotted in figure 4. The effects in the two-way mixed-model ANOVAs were significant at both temperatures in most cases (tables 1 and 2), including subtle directional asymmetries of wing size in all samples but males reared at 18ºC (mean centroid sizes for females at 18ºC: $\overline{L}$ = 0.9877 mm, $\overline{R}$ = 0.9860 mm; for males at 18ºC: $\overline{L}$ = 0.8934, $\overline{R}$ = 0.8926; females 23ºC: $\overline{L}$ = 0.9118, $\overline{R}$ = 0.9069; males 23ºC: $\overline{L}$ = 0.8123, $\overline{R}$ = 0.8092). DA was consistently higher at the warmest (and suboptimal) temperature and, in addition, there was some indication of genetic variation for wing size DA in males at 23ºC.

Size variation among individuals accounted for about 96% of the total variation. The intraclass correlation for size

$$\left[\frac{s^{2}_{g}\,(\mathrm{CS})}{s^{2}_{g}(\mathrm{CS}) + s^{2}_{w}(\mathrm{CS})}\right],$$

which estimates the fraction of the total phenotypic variance due to among-lines genetic differences in O_{st} gene arrangements, was higher at 18ºC (~59%) than at 23ºC (~31%) in both sexes. The corresponding interaction components $[s^{2}_{I\times S}\,(\mathrm{CS})]$ provided unbiased estimates of fluctuating asymmetry (index FA10 in Palmer 1994) in those

samples where genetic variation for DA was absent (see Santos 2002). Analyses of variance to test for genetic components of size FA (using index FA1 in Palmer 1994) only rendered statistically significant results in the males sample at 23ºC ($F_{4,25} = 3.89$, $P < 0.05$). However, this result should be taken with caution because of the biasing effect introduced by the presence of genetic variation in DA.

A close inspection of tables 1 and 2 suggests that wing size asymmetries (DA and FA) differ between temperatures, with DA being higher at the warmest temperature in both sexes but FA showing a contrasting pattern according to sex. The results cannot be ascribed to the different numbers of lines used in the experiments because similar patterns were observed when analyses were performed on the five isochromosomal lines that were common to both temperatures (results not shown). To test for the significance of the observed differences in FA between temperatures an *F*-test comparing the corresponding $s^2_w(DA_{CS})$ values in tables 1 and 2 is appropriate since those variance components provide unbiased estimates of FA (recall that $s^2_w(DA) \approx s^2_{I\times S}$ when there is no genetic variation for DA). Approximate degrees of freedom for $s^2_w(DA_{CS})$ are

$$\frac{(MS_w - MS_m)^2}{\left[\frac{(MS_w)^2}{df_w} + \frac{(MS_m)^2}{df_m}\right]},$$

where the MSs and dfs are the corresponding mean squares and degrees of freedom for 'within (I×S)' and 'measurement error', respectively. For females FA was indeed higher at 23ºC ($F_{19,55} = 2.13$, $P < 0.05$), but no significant difference was detected for males ($F_{63,7} = 2.88$, $P > 0.05$). Overall, the results point to an increase of wing size asymmetry at suboptimal temperature but a transition from FA to DA seems to happen in males (see Graham *et al*. 2003; and below).

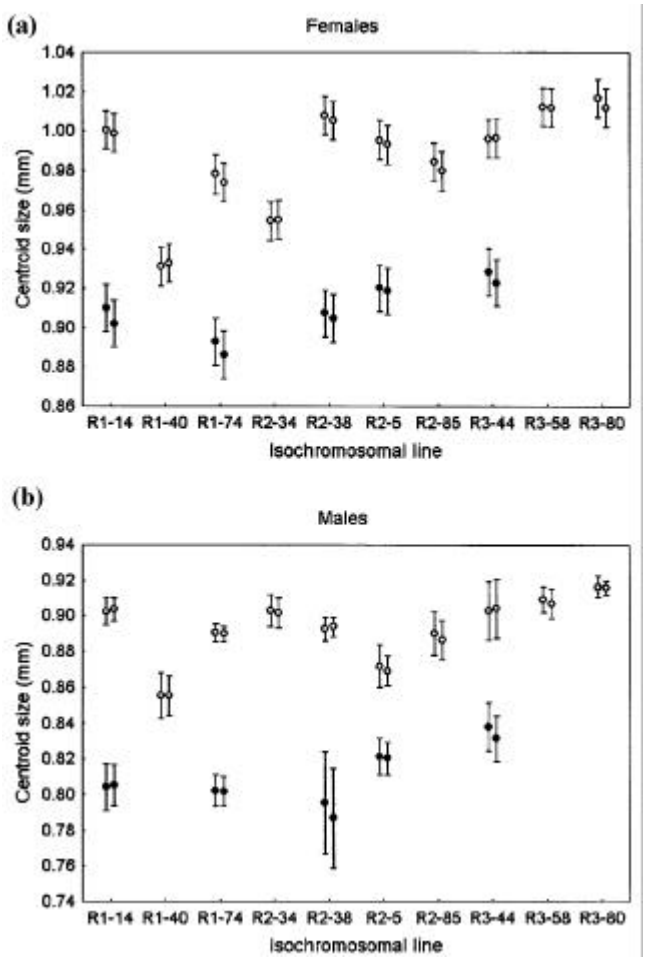

Figure 4. Averages (± 95% confidence intervals) of the centroid sizes (in a normalized form; see Dryden and Mardia 1998, p. 24) for the left and right wings, respectively, of O_{st} isochromosomal lines of *D. subobscura* reared at 18ºC (open circles) and 23ºC (closed circles).

Causal components of variation and asymmetry in wing shape

***Procrustes ANOVAs*:** The Procrustes ANOVAs for shape variation are also shown in tables 1 and 2. The main conclusions from the two-way mixed model are about the same as those obtained for centroid sizes but some differences are worth noticing. Thus shape variation among individuals accounted for less than 60% of the total variation. The intraclass correlation for shape, i.e.

$$\left(\frac{s^2_g(WS)}{s^2_g(WS) + s^2_w(WS)}\right),$$

which allows extracting here a univariate (but biased; see below) estimate of the proportion of shape variation accounted for by O_{st} gene arrangements, was lower at 18ºC (~24%) than at 23ºC (~43%). On the other hand, no DA was detected for overall shape and this clearly disagrees with previous claims on the putative biological significance of directional asymmetry in wing shape (Klingenberg *et al*. 1998; Klingenberg and Zaklan 2000).

Since shape is inherently multidimensional in nature, the amount of phenotypic and genetic variation of shape can best be assessed by examining the eigenvalues of the phenotypic (**P**) and genetic (**G**) variance–covariance matrices (Klingenberg and Leamy 2001). For each group, the **P** matrix here is simply the multivariate extension of the between-lines plus the within-lines variance–covariance components, and the **G** matrix is the multivariate extension of the between-lines component. The amounts of variation associated with the different dimensions in shape space are shown in figure 5. In all cases much of the variation was concentrated in the first few PCs. The ratios of the total variance of the **G** matrix to the total variance of the **P** matrix were 0.003 for females at 18ºC, 0.009 for males at 18ºC, 0.195 for females at 23ºC, and 0.171 for males at 23ºC. However, as stressed by Klingenberg and

Leamy (2001), these ratios cannot be interpreted as an estimate of the heritability of shape because the concept of heritability has no direct equivalent in the multivariate context. A better alternative would be to obtain the eigenvalues of the $\mathbf{GP}^{-}$ matrix, where $\mathbf{P}^{-}$ is the Moore–Penrose generalized inverse of **P**, to assess the range of heritabilities for different shape variables. However, we have used very few lines and the estimates were subject to large sampling errors.

In contrast to what had been found for wing size, temperature did not seem to have any effect on wing shape asymmetries because no significant differences between temperatures were detected for wing shape FAs (females: $F = 1.072$, $P > 0.05$; males: $F = 1.116$, $P > 0.05$). This conclusion was reached from permutation tests (after 10,000 random permutations) because degrees of freedom in Procrustes ANOVAs are not usually independent (see above). These tests can be easily performed by noticing

Table 1. ANOVAs for assessing measurement error, directional asymmetry (sides effect), fluctuating asymmetry (individuals × sides interaction effect), and genetic components of the trait (s^2_g) and DA of the trait (s^2_g (DA)) in O_{st} isochromosomal lines of *D. subobscura* reared at 18ºC. CS refers to centroid size (values in pixels^2; 1 mm = 144 pixels), and WS to wing shape (all values × 10^4). Statistical significance for WS was determined from separate permutation tests for each effect (see text for details).

Trait	Source of variation	d.f.	Sum of squares	Mean square	Variance component	Estimate
(a) Females						
CS	Individuals (I)	99	8740.87	88.292***		
	Lines	9	5195.83	577.315***	s^2_g (CS)	13.4481
	Within	90	3545.04	39.389***	s^2_w (CS)	9.7996
	Sides (S)	1	6.28	6.278*		
	I × S	99	91.60	0.925***	$s^2_{I\times S}$ (CS)	0.3672
	Lines	9	10.06	1.118$^{n.s.}$	s^2_g (DA_{CS})	0.0212
	Within	90	81.54	0.906***	s^2_w (DA_{CS})	0.3576
	Measurement error	200	38.16	0.191	s^2_m (CS)	0.1908
WS	Individuals (I)	2178	1680.026	0.771***		
	Lines	198	459.746	2.322***	s^2_g (WS)	0.0426
	Within	1980	1220.281	0.616***	s^2_w (WS)	0.1229
	Sides (S)	22	19.383	0.881$^{n.s.}$		
	I × S	2178	876.131	0.402***	$s^2_{I\times S}$ (WS)	0.1388
	Lines	198	56.252	0.284$^{n.s.}$	s^2_g (DA_{WS})	– 0.0130
	Within	1980	819.878	0.414***	s^2_w (DA_{WS})	0.1447
	Measurement error	4400	548.805	0.125	s^2_m (WS)	0.1247
(b) Males						
CS	Individuals (I)	99	4147.06	41.889***		
	Lines	9	2512.14	279.127***	s^2_g (CS)	6.5240
	Within	90	1634.91	18.166***	s^2_w (CS)	4.4925
	Sides (S)	1	1.18	1.184		
	I × S	99	118.49	1.197***	$s^2_{I\times S}$ (CS)	0.5006
	Lines	9	5.39	0.599$^{n.s.}$	s^2_g (DA_{CS})	– 0.0657
	Within	90	113.09	1.257***	s^2_w (DA_{CS})	0.5304
	Measurement error	200	39.14	0.196	s^2_m (CS)	0.1957
WS	Individuals (I)	2178	1630.068	0.748***		
	Lines	198	418.633	2.114***	s^2_g (WS)	0.0376
	Within	1980	1211.435	0.612***	s^2_w (WS)	0.1307
	Sides (S)	22	7.493	0.341$^{n.s.}$		
	I × S	2178	1061.856	0.488***	$s^2_{I\times S}$ (WS)	0.1993
	Lines	198	92.433	0.467$^{n.s.}$	s^2_g (DA_{WS})	– 0.0023
	Within	1980	969.423	0.490***	s^2_w (DA_{WS})	0.2003
	Measurement error	4400	391.298	0.089	s^2_m (WS)	0.0889

$^{n.s.}P > 0.05$; $*P < 0.05$; $***P < 0.001$.

that the mean square for the I×S interaction terms in the Procrustes ANOVAs are simply the sum of $Var_{(L-R)}$ (index FA4 in Palmer 1994) for each *x* and *y* coordinates of the corresponding aligned configurations divided by the shape dimension (i.e. 2*p*–4; see above), and we can contrast the observed ratio of FA4 indexes with the vector of randomized ratios.

Those permutation tests do not, however, take into account the covariances among the coordinates of superimposed landmark configurations. An easy alternative is to perform a MANOVA with temperature and sex as fixed effects, and the unsigned $|L - R|$ estimates for each *x* and *y* coordinates of the aligned configurations as dependent variables. This MANOVA test showed significant differences for temperature (Hotelling's $T^2 = 0.182$; $P = 0.034$), with sex ($T^2 = 0.067$; $P = 0.944$) and temperature × sex interaction ($T^2 = 0.123$; $P = 0.347$) effects being statistically non-significant. It seems therefore that wing shape FA does slightly increase at the suboptimal and warmer temperature.

Tables 3 and 4 give the variance components from Procrustes ANOVAs when apportioned by landmarks; however, we emphasize here again that these values do

Table 2. Same as in table 1, for O_{st} isochromosomal lines of *D. subobscura* reared at 23ºC.

Trait	Source of variation	d.f.	Sum of squares	Mean square	Variance component	Estimate
(a) Females						
CS	Individuals (I)	29	1237.03	42.656***		
	Lines	4	390.14	97.535*	s_g^2 (CS)	2.6525
	Within	25	846.89	33.876***	s_w^2 (CS)	8.4098
	Sides (S)	1	15.07	15.075**		
	I × S	29	47.38	1.634***	$s^2_{I\times S}$ (CS)	0.6986
	Lines	4	3.38	0.845$^{n.s.}$	s_g^2 (DA_{CS})	– 0.1525
	Within	25	44.00	1.760***	s_w^2 (DA_{CS})	0.7617
	Measurement error	60	14.19	0.237	s_m^2 (CS)	0.2366
WS	Individuals (I)	638	564.236	0.884***		
	Lines	88	233.973	2.659**	s_g^2 (WS)	0.0858
	Within	550	330.263	0.600***	s_w^2 (WS)	0.1194
	Sides (S)	22	18.515	0.842$^{n.s.}$		
	I × S	638	239.310	0.375***	$s^2_{I\times S}$ (WS)	0.1261
	Lines	88	25.508	0.290$^{n.s.}$	s_g^2 (DA_{WS})	– 0.0165
	Within	550	213.803	0.389***	s_w^2 (DA_{WS})	0.1329
	Measurement error	1320	162.320	0.123	s_m^2 (WS)	0.1230
(b) Males						
CS	Individuals (I)	29	1374.18	47.385***		
	Lines	4	583.94	145.984**	s_g^2 (CS)	4.7656
	Within	25	790.24	31.610***	s_w^2 (CS)	7.8269
	Sides (S)	1	5.97	5.968**		
	I × S	29	25.72	0.887***	$s^2_{I\times S}$ (CS)	0.2925
	Lines	4	8.97	2.244*	s_g^2 (DA_{CS})	0.2623
	Within	25	16.75	0.670**	s_w^2 (DA_{CS})	0.1840
	Measurement error	60	18.12	0.302	s_m^2 (CS)	0.3021
WS	Individuals (I)	638	462.873	0.726***		
	Lines	88	184.569	2.097**	s_g^2 (WS)	0.0663
	Within	550	278.304	0.506***	s_w^2 (WS)	0.0817
	Sides (S)	22	23.279	1.058$^{n.s.}$		
	I × S	638	278.756	0.437***	$s^2_{I\times S}$ (WS)	0.1288
	Lines	88	38.697	0.440$^{n.s.}$	s_g^2 (DA_{WS})	0.0005
	Within	550	240.059	0.436***	s_w^2 (DA_{WS})	0.1285
	Measurement error	1320	236.808	0.179	s_m^2 (WS)	0.1794

$^{n.s.}P > 0.05$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

not take into account the covariances among coordinates. (In addition, it should be stressed that variance components for shape effects were adjusted in each case by dividing for the number of measurements taken to make them comparable to results in tables 1 and 2 (see Santos 2001, 2002).) Landmarks 3 and 6 had the largest effects, but the among-females variation for landmark 6 seems to be mainly environmental. On the other hand, landmark 1 had relatively low amounts of variability among individual flies. DA appears to be present for landmarks 11 and 12 in females at both temperatures, which suggests that the position of the anterior cross-vein varies between sides. After plotting the Procrustes grand mean shapes of both wings in females samples it became apparent that the location of that vein was slightly more distal in the right wings. However, overall shape variation mainly displays FA because side effects were generally nonsignificant.

***Patterns of shape variation*:** We will focus here mainly on individual and among-O_{st}-isochromosomal-lines variation. PCA of overall shape variation yielded results that were fairly consistent with those previously obtained from our thermal selection stocks (Santos *et al*. 2004). The first three PCs for both sexes and temperatures are plotted in figure 6. The relatively high level of variability explained by a few PCs clearly suggests strong dependencies among landmarks and, hence, the isotropic model does not seem to hold (see Dryden and Mardia 1998, p. 97). The direction of PCs is arbitrary and all movements can be simultaneously reversed by 180º. Some contrasting patterns between temperatures are perceptible in both sexes, mainly involving the shifts of those landmarks that define the positions of the cross-veins. For instance, the shifts of the anterior and posterior cross-veins appear to be rather independent of each other at 18ºC but not at 23ºC. These results suggest that wing shape in *Drosophila* may not be as strongly resistant to environmental influences as previously thought (see Birdsall *et al*. 2000).

The features of among-lines variation associated with the dominant PCs are plotted in figure 7. The dominance of PC1 was also linked to the variability in outer landmarks 3, 4 and 6. As before, some differences between temperatures are clearly appreciated, which suggests temperature × line interactions. For instance, shifts of landmark 9 are only apparent at 23ºC for both sexes. These temperature effects on wing shape genetic variation are quite

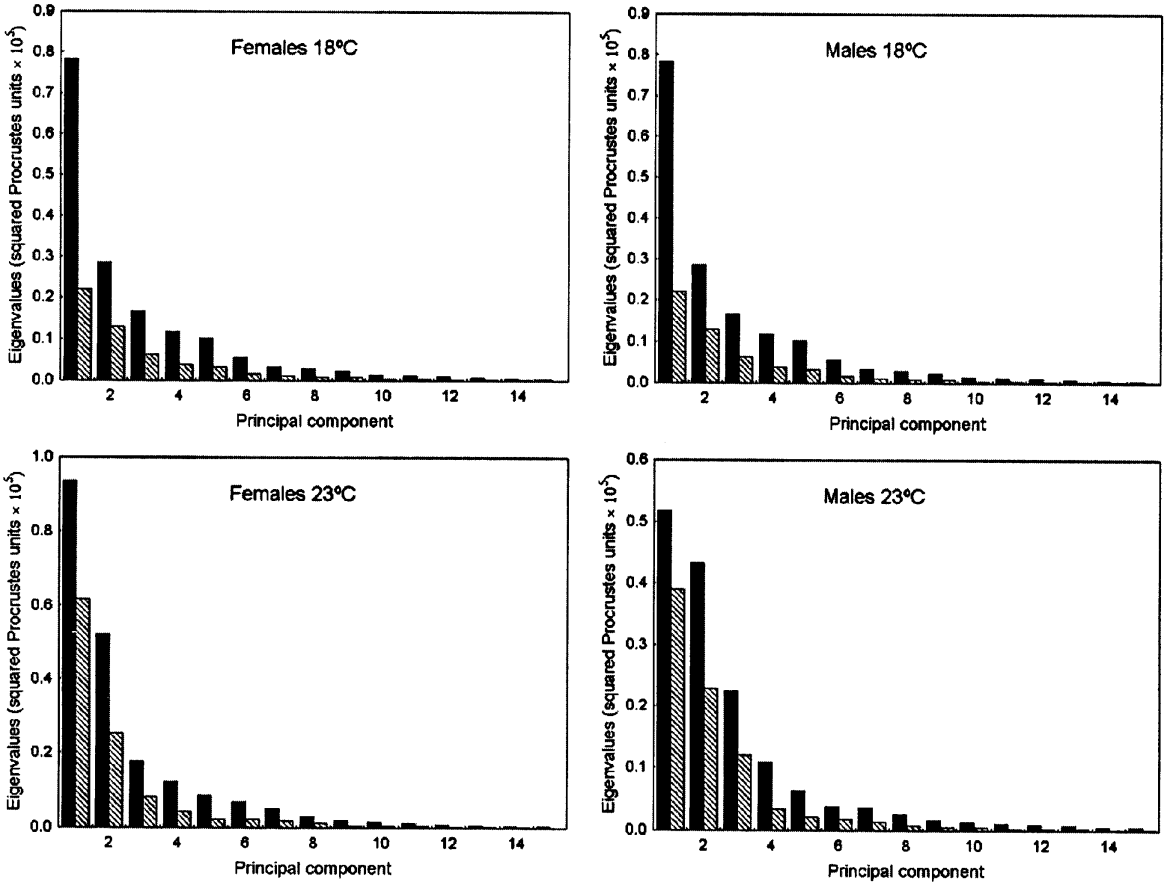

Figure 5. Eigenvalues of the phenotypic (solid bars) and genetic (hatched bars) covariance matrices of wing shape. Note that these are only the first 15 eigenvalues out of 22 shape dimensions, but together make up more than 98% of the total shape variation.

noticeable from the Procrustes three-way mixed ANOVAs discussed below.

Within each group, the congruence of landmark displacements between effects was assessed by comparing the absolute angles between respective eigenvectors. The PC1 of line variation was generally different from the PC1 of FA (females: 72.6º at 18ºC and 36.7º at 23ºC; males: 68.3º at 18ºC and 80.8º at 23ºC); only the association for females at 23ºC was highly significant (recall that the 0.1% and 2.5% quantiles of the distribution of angles between 22-dimensional random vectors were 50.3º and 62.3º, respectively). A similar pattern was found for the association between the PC1s of line and measurement error. However, PC1s of FA and measurement error were quite similar to each other (females: 4.3º at 18ºC and 11.4º at 23ºC; males: 3.6º at 18ºC and 10.8º at 23ºC).

Considering the variation among lines between groups (by using only those five lines that were common at both

Table 3. Variance components ($\times 10^8$) for the effects of each landmark in the Procrustes ANOVAs in O_{st} isochromosomal lines of *D. subobscura* reared at 18ºC (see text for details).

	Landmark												
	1	2	3	4	5	6	7	8	9	10	11	12	13
(a) Females													
Individuals (I)	680***	2496***	6999***	2405***	1147***	7516***	4536***	945***	983***	1490***	2215***	2316***	1838***
Lines	198***	471***	1441***	1043***	287***	552	999***	227***	255***	888***	1075***	1377***	569***
Within	500***	2068***	5690***	1457***	886***	7014***	3628***	738***	751***	682***	1238***	1065***	1320***
Sides (S)	0	0	125	6	13	221	101	7	6	16	37**	27**	0
I × S	402***	2336***	7366***	1554***	732***	9964***	4196***	799***	616***	859***	520***	357***	829***
Lines	0	0	0	0	0	0	0	0	0	0	43	0	0
Within	431***	2396***	7760***	1636***	747***	10438***	4382***	803***	630***	905***	500***	360***	841***
Measurement	871	2977	5954	1406	642	8476	3644	774	816	967	344	169	402
(b) Males													
Individuals (I)	695***	2486***	6715***	2143***	1409***	8023***	4385***	892***	1082***	1197***	2574***	2700***	1971***
Lines	170***	118	404	636***	543***	645*	1009***	291***	410***	512***	1540***	1584***	401***
Within	540***	2379***	6348***	1565***	916***	7436***	3468***	627***	709***	731***	1174***	1260***	1606***
Sides (S)	18	111*	0	0	2	0	0	0	0	47*	8	0	0
I × S	620***	4106***	9896***	2309***	1161***	14105***	6709***	848***	821***	1168***	516***	484***	1103***
Lines	0	0	78	0	61	0	0	20	0	111	0	30	0
Within	647***	4140***	9861***	2373***	1133***	14276***	6721***	839***	840***	1118***	536***	471***	1119***
Measurement	747	2362	4053	945	476	5402	2287	725	684	1075	281	207	321

**P* <0.05; ***P* <0.01; ****P* <0.001 (no Bonferroni correction was applied).

Table 4. Variance components ($\times 10^8$) for the effects of each landmark in the Procrustes ANOVAs in O_{st} isochromosomal lines of *D. subobscura* reared at 23ºC (see text for details).

	Landmark												
	1	2	3	4	5	6	7	8	9	10	11	12	13
(a) Females													
Individuals (I)	804***	3460***	9903***	3044***	1119***	8362***	5843***	1170***	1227***	1060***	1860***	2249***	1778***
Lines	121	1658***	7354***	875**	190	1562	3816***	537***	222	375**	476*	559*	1123***
Within	704***	2088***	3817***	2319***	961***	7069***	2685***	726***	1043***	750***	1466***	1786***	849***
Sides (S)	0	0	407	79	58*	561	466*	24	23	0	73*	66*	0
I × S	509***	3507***	4799***	1678***	455***	7831***	4587***	858***	873***	831***	603***	439***	764***
Lines	287*	0	0	0	0	0	0	82	67	0	0	0	266
Within	390***	3709***	5036***	1821***	460***	8208***	5121***	825***	845***	980***	685***	501***	654***
Measurement	736	2694	5891	1680	639	8320	3692	849	772	840	305	210	425
(b) Males													
Individuals (I)	505***	1405***	6341***	3170***	1057***	4386***	4991***	716***	959***	1010***	1122***	1713***	2659***
Lines	126	611**	2918**	2797***	519***	2398**	2494***	468***	293**	891***	180	238	652*
Within	401***	899**	3926***	856***	628***	2401**	2927***	329***	716***	272*	973***	1516***	2119***
Sides (S)	16	23	609	185	81	1042*	113	45	35	117	12	32	0
I × S	209	2344***	7757***	1503***	576***	7456***	4057***	849***	660***	1469***	358***	370***	718***
Lines	0	287	0	0	144	1099	0	0	224	0	32	0	0
Within	257	2225***	7766***	1535***	516**	7001***	4354***	886***	567***	1616***	345***	444***	766***
Measurement	1236	3612	8140	2609	1144	12411	5038	1012	799	1990	529	342	608

**P* <0.05; ***P* <0.01; ****P* <0.001 (no Bonferroni correction was applied).

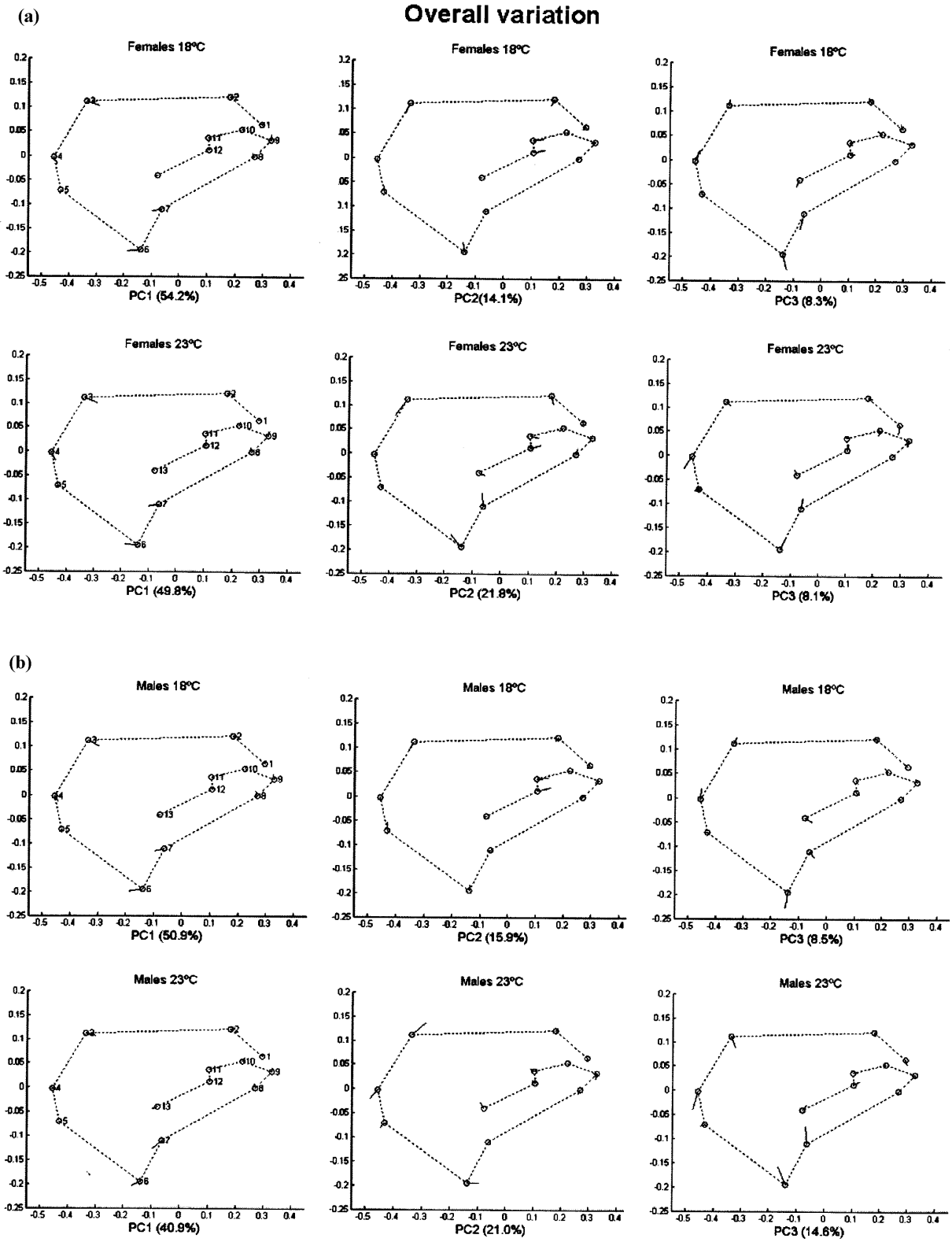

Figure 6. Principal components of the covariance patterns in landmark shifts due to overall variation for females (a) and males (b). The PC coefficients are shown as a solid line originating at the mean location of the landmark (open circles) and ending at the location to which the landmark would move at +6 (PC1), +10 (PC2) and +12 (PC3) standard deviations (obviously an exaggeration of the variation in the dataset). The proportion of total variation accounted for by each PC is given in brackets.

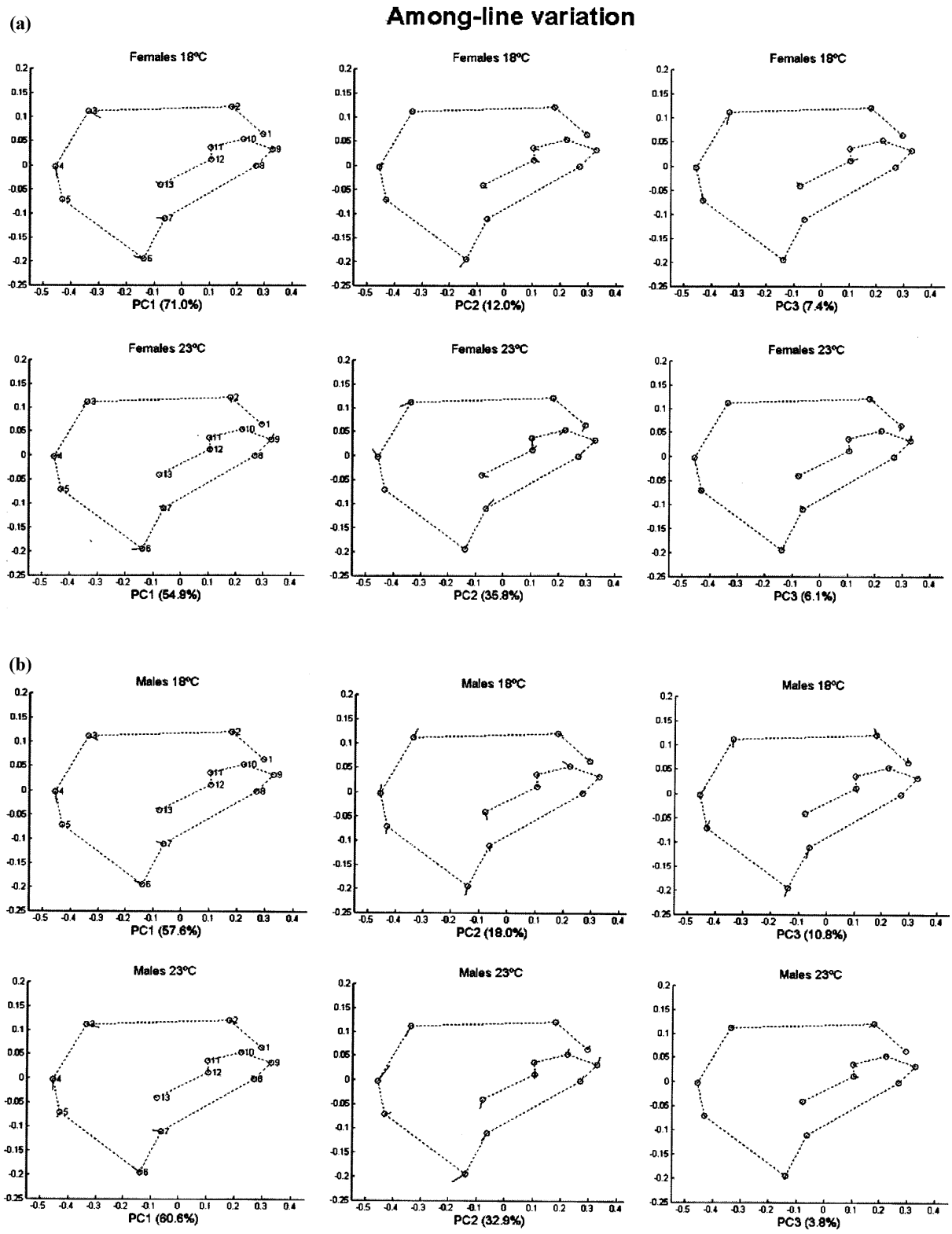

Figure 7. Principal components of the covariance patterns in landmark shifts due to among-lines variation for females (a) and males (b). The end points of the solid lines are at locations displaced +6 (PC1), +10 (PC2) and +12 (PC3) standard deviations from the mean configuration.

temperatures), the angles between the two PC1s were 44.8º and 75.1º, and those between the two PC2s were 73.5° and 89.8º for females and males, respectively. These low associations (only significant for the PC1s of females) were somewhat surprising and suggest important temperature effects for shape during the developmental process (see below). On the other hand, the associations concerning FA were strong since the angles between the PC1s were 10.2º and 10.5º for females and males, respectively.

Temperature effects on wing size and shape

Three-way mixed ANOVAs as described in Materials and methods and summarized in table 5 were used to partition the effects of temperature, sex and O_{st} isochromosomal line, as well as all possible interaction terms. As expected, all three main effects were highly significant for centroid size, and there was also indication that the different lines responded to temperature to different extents. Sex and line effects were also highly significant for wing shape, but no temperature differences were detected for overall shape (notice, however, that $P \sim 0.07$). Nevertheless, there was a highly significant temperature × line interaction, thus suggesting that temperature had consistent but somewhat different effects on overall wing shape depending on genotype.

When all effects were apportioned by landmarks in a similar way to that described for the two-way mixed-model ANOVAs (see tables 3 and 4), temperature was found to have the largest effect on landmarks 9, 1, 10, 11 and 12; whereas temperature × line interaction mainly affected landmarks 12 and 11 (which define the position of the anterior cross-vein) and, to a smaller extent, landmarks 7 and 13 (which define the position of the posterior cross-vein; results not shown). In conclusion, wing shape is clearly under genetic control in *D. subobscura* (as had also been found earlier in *D. melanogaster*; see Weber 1990, 1992; Gilchrist and Partridge 1999, 2001; Weber *et al*. 1999, 2001; Birdsall *et al*. 2000; Gilchrist *et al*. 2000; Zimmerman *et al*. 2000), and does not seem to be as strongly resistant to temperature effects as claimed (Birdsall *et al*. 2000).

Discussion

We have used the recently developed methodologies in geometric morphometrics to analyse shape configurations of landmarks within the standard statistical framework employed in studies of bilateral asymmetries, and have extended these methods to partition the individual variation and the variation in asymmetries into genetic and environmental causal components (Palmer 1994; Klingenberg and McIntyre 1998; Klingenberg and Zaklan 2000; Santos 2002). We have exploited the opportunity that *D. subobscura* offers for obtaining genetically homogeneous isochromosomal lines, and the work is being extended to include more chromosomal classes besides O_{st} and to performing line crosses to see whether or not increased FA is associated with homozygosity or is genotype dependent or both (some results suggest that heterozygous flies are more homeostatic than their homozygous counter-

Table 5. Three-way ANOVAs of wing size (CS: values in pixels^2; 1 mm = 144 pixels) and shape (WS: all values $\times 10^4$) by temperature (fixed effect) and sex (fixed) considering those five O_{st} isochromosomal lines (random) that were tested at both temperatures. Statistical significance for WS was determined from separate permutation tests for each effect.

Trait	Source of variation	d.f.	SS	MS	*F*
CS	Temperature (T)	1	4447	4446.9	82.81***
	Sex (S)	1	6712	6711.5	499.44***
	Line (L)	4	224	56.0	12.48***
	T×S	1	25	25.3	2.75
	T×L	4	215	53.7	11.98***
	S×L	4	54	13.4	3.00*
	T×S×L	4	37	9.2	2.06
	Error	140	628	4.5	
WS	Temperature (T)	22	55.66	2.530	2.99
	Sex (S)	22	43.16	1.962	17.46***
	Line (L)	88	141.07	1.603	7.41***
	T×S	22	8.42	0.383	3.26*
	T×L	88	74.51	0.847	3.92***
	S×L	88	9.89	0.112	0.52
	T×S×L	88	10.34	0.118	0.54
	Error	3080	665.93	0.216	

*P <0.05; ***P <0.001.

parts; see Pfriem 1983). Here we attempt to interpret the initial results.

Not surprisingly the intraclass correlations for wing size and shape were relatively high but within the range of the heritabilities reported in the *Drosophila* literature for these traits (e.g. Roff and Mousseau 1987; Weber 1990, 1992; Leibowitz *et al*. 1995; Birdsall *et al*. 2000). However, contrasting patterns were observed for CS (centroid size) and WS (wing shape) intraclass correlations according to developmental temperature, and it is worth noting that the higher intraclass correlation for WS at 23ºC was mainly brought about by an increase in the genetic component (c.f. tables 1 and 2). Hoffmann and Merilä (1999) have recently reviewed some *Drosophila* studies that point to an increase of heritability under unfavourable thermal conditions, but results are generally inconsistent and in most cases the works have focussed on size-related traits. In addition, heritability can be a misleading concept because it is the ratio of two variances in a given environment. In the case of WS, it is quite clear that this trait is more resistant to environmental influences than size-related traits (Weber 1990; Birdsall *et al*. 2000; table 5 here), and the observed increase of genetic variation at the suboptimal temperature of 23ºC could be made consistent with the idea that WS has a past history of selection for canalization (Waddington 1961; Hoffmann and Merilä 1999). In any case, line effects were quite important for WS and it is likely that these effects would be larger if different chromosomal classes in addition to O_{st} were included in the analyses. Since strong and consistent latitudinal clines for chromosome arrangement frequencies are observed worldwide in *D. subobscura* (Krimbas and Loukas 1980; Prevosti *et al*. 1985, 1988; Menozzi and Krimbas 1992; Balanyà *et al*. 2003), together with rapid microevolution for the O chromosomal inversion polymorphism as a putative response to shifts in environmental temperature (Rodríguez-Trelles *et al*. 1996; Rodríguez-Trelles and Rodríguez 1998), it would be very interesting to see how particular aspects of wing shape are affected by different chromosome O arrangements.

Some qualitatively different patterns were also observed for CS and WS asymmetries. Thus a significant level of DA was generally found for centroid size with left wings slightly bigger than the right ones (figure 4). This result closely agrees with previous findings in *D. melanogaster* (Klingenberg *et al*. 1998; Klingenberg and Zaklan 2000), which apparently raises sound warnings against the conventional wisdom in *Drosophila* (Maynard Smith and Sondhi 1960; Coyne 1987; see Tuinstra *et al*. 1990 for a review). Conversely, no DA was found for overall wing shape and this is against some claims suggesting that DA has been evolutionarily conserved in fly wings (Klingenberg *et al*. 1998). As far as we are aware, this is the first study dealing with the putative genetic basis of wing shape DA in *Drosophila*, and the conclusion is that there seems to be no genetic variation for this trait (tables 1 and 2). Adding to the conundrum, genetic variation was detected for centroid size DA in males raised at 23ºC, together with an increase of wing size asymmetry at this suboptimal temperature and a transition from FA to DA. According to Graham *et al*. (2003), the classical linear theory of DI can successfully account for both normally distributed error distributions and leptokurtic distributions caused by admixture of individuals with different levels of DI, but cannot account for transitions between FA and DA. We are not, however, totally convinced that this is indeed the case. Thus, let us focus on centroid sizes of males at both temperatures and assume for the time being that genetic variation for DA (which has often been thought of to occur and has occasionally been found in other organisms but not in *Drosophila*; see Palmer *et al*. 1993; Palmer 1994; Leamy *et al*. 1997, 2000) is present and changes as a direct response to environmental conditions in which it is expressed. FA estimated from index FA10 ($s^2_{I\times S}$) would be biased whenever genetic variation for DA is expressed (Santos 2001, 2002; table 2b here), and signed ($L - R$) asymmetries will not necessarily have to be centred at zero (there can be, however, genetic variation for DA in traits that exhibit nonsignificant DA; see Leamy *et al*. 1997). It seems to us, therefore, that a transition from 'ideal' FA (i.e. a normal distribution of left–right scores whose mean is zero; see Palmer 1994) to a distribution showing DA could be made entirely compatible with what is already known from classical quantitative genetics.

Anyway, the important question here is to know whether or not there is genetic variation for DA in *Drosophila* as suggested by Klingenberg *et al*. (1998). Taking all the information together, what seems clear is that *Drosophila* traits do exhibit DA at least under some circumstances, although genetic variation for DA has almost never been detected. The DA asymmetry in particular aspects of shape (i.e. the slight variation in anterior cross-vein of females) is subtle and these small shifts of wing veins are unlikely to impede aerodynamic properties. Likewise DA of wing size was quite small in our samples and substantially less than nondirectional asymmetry (of the order of 4% FA) at 18ºC, but accounted for a sizeable proportion of overall wing size asymmetry at 23ºC that would likely represent a burden in flight performance. Transitions from FA to DA could be easily explained if we assumed environment-dependent genetic expression for DA but, for the time being, no definitive answer on the putative genetical basis of DA in *Drosophila* can be given. Accordingly, we parsimoniously adhere to the conventional wisdom that left and right are not distinguished in *Drosophila* development (but see Ligoxygakis *et al*. 2001).

One of the main aims of the ongoing work is to test for genetic effects on FA. No genetic variation was generally

detected for FA in our samples from O_{st} isochromosomal lines, but we have already pointed out that these are preliminary results because no crosses between lines were performed and that most genetic variation in New World colonizing populations of *D. subobscura* is likely apportioned among chromosome arrangements. Thus, bottleneck effects are quite obvious when comparing chromosome, allozyme and microsatellite diversities among original Palaearctic and colonizing populations from the New World with stronger allozyme-inversion disequilibria in the latest populations (e.g. Prevosti *et al*. 1983; Balanyà *et al*. 1994; Pascual *et al*. 2001). These disequilibria are also very strong between microsatellite alleles and chromosome O inversions; all but two isochromosomal lines used here are fixed for the same allele at two microsatellite loci on this chromosome (unpublished results). Anyhow, our initial results strongly suggest that FA only reflects developmental noise and that O_{st} chromosomes from Puerto Montt seem to exhibit similar levels of DI.

Finally, it is worth mentioning that the general lack of correspondence between line variation and FA (as assessed by using the angles between corresponding PCs) suggests that developmental processes influencing wing shape do not necessarily generate random differences between body sides, contrarily to what has been previously suggested on empirical (Klingenberg and McIntyre 1998) and theoretical (Klingenberg and Nijhout 1999) grounds. This is clearly an important problem that needs to be addressed in more detail with a larger data set than the one we have used here.

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Swift laboratory thermal evolution of wing shape (but not size) in *Drosophila subobscura* and its relationship with chromosomal inversion polymorphism

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Abstract

Latitudinal clinal variation in wing size and shape has evolved in North American populations of *Drosophila subobscura* within about 20 years since colonization. While the size cline is consistent to that found in original European populations (and globally in other *Drosophila* species), different parts of the wing have evolved on the two continents. This clearly suggests that 'chance and necessity' are simultaneously playing their roles in the process of adaptation. We report here rapid and consistent thermal evolution of wing shape (but not size) that apparently is at odds with that suggestion. Three replicated populations of *D. subobscura* derived from an outbred stock at Puerto Montt (Chile) were kept at each of three temperatures (13, 18 and 22 °C) for 1 year and have diverged for 27 generations at most. We used the methods of geometric morphometrics to study wing shape variation in both females and males from the thermal stocks, and rates of genetic divergence for wing shape were found to be as fast or even faster than those previously estimated for wing size on a continental scale. These shape changes did not follow a neat linear trend with temperature, and are associated with localized shifts of particular landmarks with some differences between sexes. Wing shape variables were found to differ in response to male genetic constitution for polymorphic chromosomal inversions, which strongly suggests that changes in gene arrangement frequencies as a response to temperature underlie the correlated changes in wing shape because of gene-inversion linkage disequilibria. In fact, we also suggest that the shape cline in North America likely predated the size cline and is consistent with the quite different evolutionary rates between inversion and size clines. These findings cast strong doubts on the supposed 'unpredictability' of the geographical cline for wing traits in *D. subobscura* North American colonizing populations.

Introduction

Patterns of morphological variation mostly involving size-related dimensions across latitudinal/altitudinal gradients are often interpreted in relation to climatic conditions, mainly temperature. In endotherms this is exemplified by Bergmann's rule: 'the smaller-sized geographical races of a species are found in the warmer parts of the range, the larger-sized races in the cooler districts' (Mayr, 1942). Geographical clines in body size, with genetically larger individuals derived from higher latitudes, have also been documented in a number of ectothermic animals, particularly insects from the genus *Drosophila* (e.g. Stalker & Carson, 1947; Prevosti, 1955; David *et al.*, 1977; Coyne & Beecham, 1987; Pegueroles *et al.*, 1995; James *et al.*, 1997; van't Land *et al.*, 1999; Huey *et al.*, 2000). Is there a Bergmann's rule in

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ectotherms (cf. Mousseau, 1997; Partridge & Coyne, 1997; Van Voorhies, 1997)? Fitness costs related to the effects of surface/volume ratio on heat loss are assumed to underlie the rule, but it is obvious that size clines in ectotherms warrant a different explanation because small insects adopt ambient temperature almost instantaneously (Stevenson, 1985).

Although temperature is not the only factor that varies with latitude, laboratory studies carried out with a number of *Drosophila* species have repeatedly observed thermal selection on body dimensions that goes in the predicted direction according to the size clines (Anderson, 1966; Powell, 1974; Cavicchi *et al.*, 1985, 1989; Partridge *et al.*, 1994a). There are, however, some potentially important shortcomings with these experiments. Thus, no replicated populations were kept in Powell's or Cavicchi's *et al.* studies and, hence, the 'random-walk' hypothesis (which claims that evolutionary rates generally do not exist) cannot be discarded (see Bookstein, 1991, pp. 393–398). However, Anderson's and Partridge's *et al.* thermal stocks were replicated but flies were maintained in population cages by regularly introducing bottles with fresh food and removing them after several weeks, a routine that does not allow control of larval densities. Crowding conditions differ between population cages maintained at different temperatures, with higher larval densities in warmer environments (L. Partridge, pers. comm., 1998). There is conflicting evidence about the importance of larval density on the evolutionary responses of body size in *Drosophila* (cf. Roper *et al.*, 1996; Santos *et al.*, 1997); however, it is well established that harmful waste products accumulate in crowded cultures (Borash *et al.*, 1998, 2000) and many of the adaptations to different levels of larval crowding (Joshi & Mueller, 1988, 1993; Guo *et al.*, 1991; Mueller *et al.*, 1993; Borash *et al.*, 1998) involve changes in larval behaviour and physiology that may impinge on other phases of the life cycle (see e.g. Joshi & Mueller, 1996; Santos, 1996; Santos *et al.*, 1997; Houle & Rowe, 2003). Therefore, although the previous studies – as well as those that have also reported evolutionary responses in other fitness-related traits in the thermal stocks (Huey *et al.*, 1991; Partridge *et al.*, 1994b; James & Partridge, 1995; Azevedo *et al.*, 1996) – do demonstrate rapid adaptations, it is by no means obvious that temperature is the main factor or that body size is the target of selection (see Bochdanovits & De Jong, 2003).

The historically Paleartic species *D. subobscura* provides a suitable model system to study the dynamics of clinal variation and the biological effects of the current greenhouse-induced increase in world temperatures (Houghton *et al.*, 2001) for a number of reasons. First, there is an extensive amount of information on the geographical distribution of chromosomal arrangements in this particularly inversion-rich species, with the so-called standard arrangements in the five (out of six considering the dot chromosome) acrocentric chromosomes increasing in frequency with latitude in European populations (Krimbas & Loukas, 1980; Menozzi & Krimbas, 1992). Most important, similar clines quickly developed (within about 7 years or ~35 generations) after the double colonization of South and North America by the species (Prevosti *et al.*, 1985, 1988), strongly supporting the hypothesis that environmental latitudinal gradients are responsible for the clines. Further support comes from several independent observations showing within-population long-term directional trends in the inversion polymorphism that correlated with expectations from the latitudinal clines (Orengo & Prevosti, 1996; Solé *et al.*, 2002), although a putative critical role played by a shift in temperatures could only be ascribed to those trends tracked for the gene arrangements of chromosome O for which relatively long time-series matching both the inversion frequencies and the variation in temperatures exist (Rodríguez-Trelles *et al.*, 1996; Rodríguez-Trelles & Rodríguez, 1998).

Secondly, parallel body size clines as the long-standing ones in native European populations (Prevosti, 1955; Misra & Reeve, 1964; Pfriem, 1983; Pegueroles *et al.*, 1995) appeared in New World populations too, although it took about 20 years after the colonization for the clines to build up (Huey *et al.*, 2000; Gilchrist *et al.*, 2001). Interestingly, the size clines are not isometric in the sense that the relative contribution of two portions of longitudinal vein IV used to measure wing length (WL) also changed with latitude but in an opposite way according to the northern hemisphere continent (i.e. Europe vs. North America). On the basis of previous studies in *D. melanogaster* (Gilchrist *et al.*, 2000) it was hypothesized that the wing shape variation in *D. subobscura* may simply represent drift around an optimum. Consistent with this idea, recent biometric and quantitative trait loci (QTL) analyses suggest that wing size and shape have a contrasting genetic architecture; the former likely being subjected to directional selection and the latter to optimizing selection and regulated largely independently of wing size, with up to 50 loci throughout the *D. melanogaster* genome having a significant and generally additive effect on wing shape as well as minor pleiotropic effects on fitness (Weber *et al.*, 1999, 2001; Zimmerman *et al.*, 2000; Gilchrist & Partridge, 2001). Birdsall *et al.* (2000) used the method of relative warps (Bookstein, 1991) to study wing shape variation in 12 inbred lines from *D. melanogaster* at 18 and 25 °C. They found that the two rearing temperatures caused differences in wing area of up to 20%, but wing shape seemed to be independent of sex and temperature effects on cell growth and density. This is important because the cellular basis of the body size cline for *D. subobscura* in North America (latitudinal variation in cell area) is different from that in Europe and South America clines (latitudinal variation in cell number; Calboli *et al.*, 2003). Therefore, the opposite latitudinal gradients for wing shape (Huey *et al.*, 2000)

do not seem to be related to the cellular bases of the clines, as suggested by the work of Birdsall *et al.* (2000).

Third and finally, Orengo & Prevosti (2002) recently presented evidence for a positive relationship between wing size and standard gene arrangement dose, an expected trend according to the latitudinal clines. However, they used two samples of wild-caught *D. subobscura* males and the likely presence of nongenetic effects on body size precludes any firm conclusion. In addition, the different evolutionary rates observed in colonizing populations when comparing chromosomal polymorphism and wing size (see above) is somewhat puzzling from that putative genetic connection. Further work is clearly needed.

We have developed a set of three replicated *D. subobscura* populations kept at three temperatures (13, 18 and 22 °C) to study the short- and long-term effects of thermal selection on chromosomal inversion polymorphism and wing size and shape. A large stock from Puerto Montt (Chile) was chosen as the base population because (1) the species was detected for the first time in America at this locality in February 1978 (Brncic *et al.*, 1981); (2) the stock harboured all polymorphic chromosome arrangements involved in the New World latitudinal clines (Prevosti *et al.*, 1988); and (3) the introduction of the species into South and North America (first detected in Port Townsend, Washington, in 1982; Beckenbach & Prevosti, 1986) was the result of a single colonizing event from a Paleartic population (Mestres *et al.*, 1992), which provides a unique opportunity to empirically test how replicated clinal patterns in nature relate to temperature. Our rearing protocol allows controlling for larval densities, thus minimizing those potentially spurious correlated responses that may arise due to other factors not related to thermal selection. Here we report the initial results showing no size differences according to thermal regimes, a probably unsurprising finding for populations that have diverged for 27 generations at most. However, the analyses of wing shape by using the shape index in Huey *et al.* (2000) and the framework of geometric morphometrics (Bookstein, 1991; Dryden & Mardia, 1998) revealed consistent and significant differences among temperatures, a somewhat unexpected finding if shape clines were historically contingent as formerly sustained (Huey *et al.*, 2000). We discuss the results in the light of available information on *Drosophila* wing shape, and provide some empirical evidence as to suggest that the highly congruent results across replicated populations are related to gene-inversion linkage disequilibria.

Materials and methods

Thermal selection stocks

The *D. subobscura* populations originated from 93 isofemale strains derived from a large outbred stock collected by Dr J. Balanyà, Dr G. W. Gilchrist, Dr R. B. Huey and Dr M. Pascual in Puerto Montt (Chile; 41°28′S) in November 1999. The isofemale lines were kept in 90-mL bottles (~30–40 breeding adults/bottle) at 18 °C for more than 1 year (~16 generations) prior to the establishment of the thermal stocks and, hence, the experimental material had likely ceased to undergo rapid adaptation to laboratory conditions (Matos *et al.*, 2000). A large outbreeding population was founded in March 2001 by randomly dumping ~25 pairs of virgin flies from each isofemale line into three Plexiglas cages ($27 \times 21 \times 16$ cm^3) and maintained at 18 °C (12 : 12 light/dark cycle). A large number of eggs were collected from these cages, and emerging adults were randomly dumped into three new cages (18 °C; 12 : 12 light/dark cycle). Eggs were sampled from these cages over 10 consecutive days and placed in 130-mL bottles (~200–250 eggs per bottle) containing 50 mL of David's killed-yeast *Drosophila* medium (David, 1962). A total of 225 bottles were set up and randomly distributed into nine groups with 24 bottles each. Three groups were allocated at 15 °C (12 : 12 light/dark cycle), three at 18 °C (12 : 12 light/dark cycle) and three at 21 °C (12 : 12 light/dark cycle). Therefore, the three replicated thermal selection stocks were established in May 2001. The extra nine bottles were used to individually cross a random sample of emerging males to three to four virgin females from the *ch-cu* marker strain in order to estimate chromosome arrangement frequencies in the initial populations. This strain is homozygous for the morphological recessive markers on the O chromosome cherry eyes (*ch*) and curled wings (*cu*) (Koske & Maynard Smith, 1954), and its genetic background is highly homogeneous and fixed for the standard gene arrangements in all major acrocentric chromosomes but chromosome O, where it is fixed for gene arrangement O_{3+4} (Lankinen & Pinsker, 1977). Whenever feasible, one F_1 female third-instar larva derived from each cross with the homozygous *ch-cu* stock was examined for its inversion loops in polythene chromosomes to determine the gene arrangements of one set of the chromosomes from the wild-type male.

After two generations of acclimatization the 15 °C stocks were transferred to the final temperature of 13 °C, and the 21 °C stocks to 22 °C. Previous laboratory observations (reviewed in Krimbas, 1993) indicate that optimal temperature for *D. subobscura* is approximately 18 °C, and that males can become sterile at 25 °C or even lower. Latitudinal clines for optimal temperatures are quite possible to occur; however, the three temperatures explored in our thermal selection stocks likely cover much of the physiologically tolerable range in this species.

All populations are maintained on a discrete generation, controlled larval crowding regime as follows. Prior to initiating a new generation, eclosed adults from the bottles are dumped into a Plexiglas cage and supplied with liberal amounts of food (two 90 mm Ø Petri dishes with *Drosophila* medium supplemented with active dried yeast) before egg collections. The number of breeding

adults per population is typically well over 1500 flies. Once females reach their peak of fecundity (~12–13 days after emergence at 13 °C, ~7–8 days at 18 °C and ~5–6 days at 22 °C) eggs are collected over a 7-day period at 13 °C, 5-day at 18 °C and 3-day at 22 °C, and placed in 130-mL bottles (~200–250 eggs per bottle) as previously described, with a total of 24 bottles per population. Generation times (eggs $t_n \rightarrow t_{n+1}$) are ~46 days at 13 °C, ~33 days at 18 °C and ~25 days at 22 °C. Consequently, the three sets of populations differ only in the temperature they experience (humidity was not strictly controlled but adult flies in the cages had continuing access to fresh and moist food).

In May 2002 (after nine generations at 13 °C, 12 generations at 18 °C, and 15 generations at 22 °C), samples from all populations were obtained by placing eggs into eight additional 130-mL bottles per population. These bottles were cultured at 18 °C and emerging adults were dumped into Plexiglas cages for egg collections. Eggs for the experiment were collected over a 48-h period by placing Petri dishes containing nonnutritive agar with a generous smear of live yeast in the cages. Larval density was controlled during culturing (100–110 eggs per bottle), and a total of 12 bottles per population were placed at 18 °C on the same incubator shelf. Therefore, the experiment was designed so that the parents of sampled flies had also been reared at the same temperature, to control for the possibility of nongenetic parental effects on offspring size (Crill *et al.*, 1996). Emerging flies were separated by sex; females stored in Eppendorf tubes with a 3 : 1 mixture of alcohol and glycerol at 4 °C, and a sample of males (125–150 males per population randomly chosen from the 12 replicated bottles) were individually crossed in vials (2 × 8 cm containing 6 mL of food) to three to four virgin females from the *ch-cu* marker strain in order to estimate chromosome arrangement frequencies as previously described. After approximately 9 days the males were removed from the vials and individually fixed in a 3 : 1 mixture of alcohol and glycerol at 4 °C.

All fly handling was done at room temperature using CO_2 anaesthesia, on flies not less than 6 h after eclosion.

Wing size and shape

Definitions

Morphometrics involves the quantitative study of form, and it is naturally understood that form consists of size and shape (Needham, 1950). An important contribution of geometric morphometrics is the clear definition of size and shape (Dryden & Mardia, 1998). Size is defined as any positive real-valued function from a landmark configuration (i.e. a set of points that can be precisely located) matrix X that satisfies the condition $g[aX] = ag[X]$ for any positive scalar a. The shape of a set of p landmark points is the geometrical information of the configuration of points that is invariant to translation, rotation and rescaling.

Wing measurements

We analysed here the wing size and shape of flies from each experimental population. Both wings were removed and mounted on microscope slides in DPX under coverslips from 100 females per population selected haphazardly from the 12 replicated bottles (see above), as well as from all males crossed to the *ch-cu* marker strain whenever information of their chromosomal arrangements was available (>100 males per population; see Table 1). All the data used here are from the left wings. Wing images were captured using a compound microscope (Zeiss Axioskop, Jena, Germany), with low power objective (2.5×) and attached video camera (Sony CCD-Iris, Tokyo, Japan), connected to a PC computer with MGI VideoWave software. Female wings were digitized

Table 1 Mean (±SD) of the basal (L1) and distal (L2) segments of wing longitudinal vein IV, and centroid size (in a normalized form; see Dryden & Mardia, 1998, p. 24) in *Drosophila subobscura* for each thermal regime and replicated population (values are given in millimetres; 1 mm = 144 pixels).

		Females				Males			
Temperature (°C)	Replicate	*n*	L1	L2	Centroid size	*n*	L1	L2	Centroid size
13	R1	100	1.521 ± 0.040	1.283 ± 0.035	1.014 ± 0.021	115	1.356 ± 0.040	1.154 ± 0.034	0.907 ± 0.019
	R2	100	1.480 ± 0.046	1.250 ± 0.037	0.986 ± 0.023	115	1.322 ± 0.040	1.125 ± 0.034	0.884 ± 0.020
	R3	100	1.479 ± 0.044	1.256 ± 0.036	0.989 ± 0.022	107	1.322 ± 0.046	1.136 ± 0.036	0.887 ± 0.022
	Total	300	1.493 ± 0.048	1.263 ± 0.038	0.996 ± 0.025	337	1.334 ± 0.045	1.138 ± 0.037	0.893 ± 0.023
18	R1	100	1.455 ± 0.052	1.247 ± 0.040	0.975 ± 0.027	134	1.297 ± 0.045	1.122 ± 0.033	0.873 ± 0.023
	R2	100	1.452 ± 0.050	1.248 ± 0.037	0.972 ± 0.027	128	1.295 ± 0.049	1.130 ± 0.033	0.873 ± 0.024
	R3	100	1.472 ± 0.055	1.262 ± 0.038	0.986 ± 0.030	132	1.326 ± 0.046	1.142 ± 0.035	0.889 ± 0.024
	Total	300	1.460 ± 0.053	1.252 ± 0.039	0.978 ± 0.028	394	1.306 ± 0.048	1.131 ± 0.035	0.878 ± 0.025
22	R1	100	1.472 ± 0.052	1.246 ± 0.039	0.985 ± 0.026	117	1.323 ± 0.041	1.139 ± 0.034	0.889 ± 0.021
	R2	100	1.478 ± 0.050	1.257 ± 0.037	0.990 ± 0.024	109	1.313 ± 0.049	1.133 ± 0.035	0.883 ± 0.025
	R3	100	1.462 ± 0.050	1.244 ± 0.038	0.979 ± 0.025	111	1.313 ± 0.042	1.136 ± 0.037	0.884 ± 0.019
	Total	300	1.471 ± 0.051	1.249 ± 0.038	0.984 ± 0.025	337	1.317 ± 0.044	1.136 ± 0.035	0.885 ± 0.022

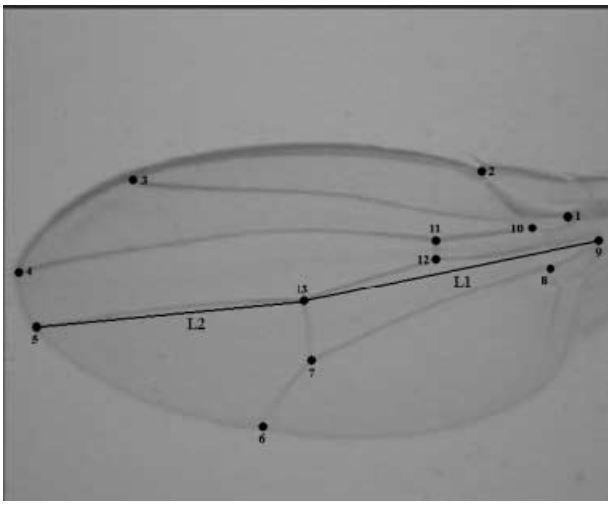

Fig. 1 Image of *Drosophila subobscura* left wing indicating the 13 landmarks used in this work. The lengths of the proximal (L1) and distal (L2) segments of longitudinal vein IV were calculated as the linear distance between landmarks 9 and13, and landmarks 13 and 5, respectively.

by M.S. and male wings by P.F.I., but all wings were measured by one of us (M.S.). Calibration of the optical system was checked at each session. *x* and *y* coordinates of 13 morphological landmarks (Fig. 1) were recorded for each wing using the image processing and analysis program for the IBM PC Scion Image (based on the NIH-Image for Macintosh and available at http://www.scioncorp.com). All landmarks are at the intersections of wing veins or at points where veins reach the wing margin and are easy to locate precisely, and can therefore be considered type 1 landmarks according to Bookstein (1991), pp. 63–67) or anatomical landmarks according to Dryden & Mardia (1998), p. 3). Using the original landmark coordinates we followed Robertson & Reeve (1952) and Prevosti (1955) by calculating WL as the combined lengths of the basal (i.e. the Euclidean distance between landmarks 9 and 13 and labelled as L1) and distal (Euclidean distance between landmarks 13 and 5 and labelled as L2) segments of longitudinal vein IV. These linear measurements have been used to study size and shape clines in *D. subobscura* (e.g. Pegueroles *et al.*, 1995; Huey *et al.*, 2000; Gilchrist *et al.*, 2001).

Procrustes analysis

Procrustes methods allow comparing configurations of landmarks by optimally superimposing (according to a least-squares criterion) homologous landmarks in two or more specimens to achieve an overall best fit (Rohlf, 1990, 1999; Rohlf & Slice, 1990; Klingenberg & McIntyre, 1998). When several objects (e.g. wings) are fitted using Procrustes superimposition (as was done in the present work) the method has been called 'generalized Procrustes analysis' (GPA) (see Dryden & Mardia, 1998, pp. 44–47). There has been some controversy on the relative merits of GPA over alternative approaches, but Rohlf (2000, 2003) has shown that the mean shape in a population can be reliably estimated using GPA. In brief, the procedure can be described as follows (Rohlf & Slice, 1990; Bookstein, 1996): (1) shift the *x* and *y* coordinates to the origin (0, 0) and scale the configurations to unit centroid size (defined as the square root of the sum of squared distances of a set of landmarks from their centroid or, equivalently, the square root of the sum of the variances of the landmarks about that centroid in *x* and *y* directions; Slice *et al.*, 1996); (2) rotate the configurations against a single reference specimen to achieve an optimal fit of corresponding landmarks; (3) a single overall consensus configuration is computed as the average coordinates of corresponding landmarks in the aligned configurations; (4) repeat steps 2 and 3 to minimize the sum of the squared distances between the landmarks of all objects in the sample and the corresponding landmarks of the consensus configurations. The final step was done without additional scaling and, consequently, we performed a partial Procrustes fit according to Dryden & Mardia (1998); see also Rohlf, 1999). [Rescaling the coordinates of each configuration by the scaling option, $1/\cos(\rho)$ (see Rohlf, 1999) would make very little difference, in the order of $\sim$0.0015 and $\sim$0.0004% of the shape variation in the female and male data sets, respectively]. The variation in the landmark coordinates that remains after Procrustes superimposition is a complete and nonredundant description of the variation in shape, and the usual linear multivariate methods focus on these coordinates (see below).

In this work we used MATLAB (V.5 and V.6; The MathWorks, Inc. 1998, 2002) for morphometric analyses, and results were checked with the 'tps' series of programs by J. F. Rohlf (available at http://life.bio.sunysb.edu/morph). Some helpful functions in morphometrics from the MATLAB toolboxes Res5 and Res6 developed by R. E. Strauss were also used (available at http://www.biol.ttu.edu/Strauss/Matlab/matlab.htm).

Statistical analyses

The unit of analysis here is the population, and the three replicated populations (R1, R2 and R3) of each thermal stock were treated as a random factor nested within experimental temperature (13, 18 and 22 °C), which was a fixed effect in the ANOVAS (see Sokal & Rohlf, 1981).

Allometry

To test for size effects on shape variation we carried out multivariate regressions of Procrustes coordinates on centroid size (Dryden & Mardia, 1998). These regressions generally accounted for around 4% of total Procrustes sums of squares; however, multivariate analyses using

the residuals of a regression on centroid size produced results that were qualitatively identical to those of the complete variation. Therefore, we only report the results of analyses of the total shape variation.

Procrustes ANOVA

As pointed out by Klingenberg & McIntyre (1998), calculation of Procrustes coordinates is based on the algebra of sums of squares, and the variance in the set of optimally aligned landmark configurations can be partitioned in a way analogous to the deviations from a grand mean in conventional ANOVA (Goodall, 1991). The coordinates of the Procrustes-aligned configurations are, therefore, amenable to the preceding two-level nested ANOVA model.

For each of the x and y coordinates of the aligned configurations a separated two-level nested ANOVA was run, and the resulting sums of squares for temperature, replicate and error in the Procrustes ANOVAS were obtained after summing the corresponding sums of squares across x and y coordinates of all landmarks. There are more degrees of freedom in Procrustes ANOVA than in conventional ANOVA (Goodall, 1991) because the squared deviations are summed over all the landmark coordinates. Therefore, the number of degrees of freedom is that for ordinary ANOVA times the shape dimension; namely, $2p - 4$ for two-dimensional coordinate data, where p is the number of landmarks.

To avoid making assumptions about the specific distribution of wing shapes around the mean landmark configuration we used permutation tests (Manly, 1997) for testing the statistical significance of ANOVA effects. Permutation tests also avoid the rather stringent statistical constraints of the covariance structure described by Goodall (1991) (see also Rohlf, 2000). For the two-level nested ANOVA model randomization is a two-stage process: (1) random permutations among subgroup (replicated populations) within group (experimental temperature) for the among-subgroup F-statistics; and (2) random permutations among subgroup and group for the among-group F-statistics. Each test used 10 000 random permutations of the observations.

Localized variation

In order to assess how much of shape variation was due to each landmark, we followed Klingenberg & McIntyre (1998) and decomposed the Procrustes mean squares for each effect in the two-level nested ANOVA model according to the landmarks. Thus, we summed x and y mean squares of each landmark separately and computed the variance components according to the expected mean squares (Sokal & Rohlf, 1981). Because the least-squares algorithm tends to spread variation from variable landmarks to the others, this approach should be taken cautiously if one or a few landmarks are much more variable than the rest (Chapman, 1990; Walker, 2000).

Shape variability

We used principal component analysis (PCA) (see e.g. Jolliffe, 1986) to investigate patterns of covariation in the positions of landmarks, which is a usual method in the context of shape analysis (Dryden & Mardia, 1998; Klingenberg & McIntyre, 1998; Klingenberg & Zaklan, 2000). The analyses must use covariance matrices of the coordinates of superimposed landmarks to avoid problems related to rotations of the coordinate system, and principal components coefficients can be presented graphically by drawing lines centred at the mean location of each landmark and ending at an arbitrary number of standard deviations away from that mean in the direction to which the landmark would shift.

The computer programs used for statistical data analyses were MATLAB (V.5 and V.6, The MathWorks, Inc. 1998, 2002), and the statistical software packages STATISTICA V.6 (StatSoft, Inc., 2003) and SPSS V.11 (SPSS Inc., 2001). They were run on a Pentium® 4 (1.60 GHz) PC-compatible.

Results

Wing size and shape index

The mean values for the basal (L1) and distal (L2) WLs and centroid sizes were calculated for females and males from each population (Table 1). Flies were considerably larger than the F_2 offspring from a wild sample collected in 1986 at the same locality of Puerto Montt and raised under similar conditions of food and temperature (see Pegueroles *et al.*, 1995). Females were approximately 11% bigger than males, and average sizes for the 13 °C thermal selection regime were slightly bigger than those at warmer temperatures. There was, however, substantial variation among replicated populations and the two-level nested ANOVAS did not detect any significant effect of thermal selection regimes on WL or centroid size. In addition, there was no indication of a linear trend between size and temperature (Table 2).

However, significant effects of thermal selection regimes (but not replicates) were observed for the length of L1 relative to total WL, and the patterns were similar in both sexes (Fig. 2). Nonlinear (deviation) effects were significant and, therefore, this shape index did not show a neat linear trend with temperature (Table 2). It is worth mentioning that *post hoc* comparisons for females only detected statistically significant differences when contrasting 18 °C vs. 13 °C or 22 °C; namely, the length of L1 relative to that observed at 18 °C significantly increased at the lowest and highest temperatures in females (but not in males where the shape index was significantly higher only at the lowest temperature; analyses not shown). When globally considered, these findings could be compatible with the contrasting patterns found in the native Paleartic populations (i.e. a positive correlation between the shape index and

Table 2 Two-level nested analyses of variance for wing length [WL: as $\log_e$(L1 + L2) in pixels; 1 mm = 144 pixels], centroid size (in pixels), and shape index (basal length/wing length) [as $\log_e$(L1/WL)]. The sums of squares for the fixed factor temperature were further decomposed to test for linear (regression) and nonlinear (deviation) effects.

		WL				Centroid size				L1/WL			
Source	d.f.	SS	MS	*F*	*P*	SS	MS	*F*	*P*	SS	MS	*F*	*P*
(a) Females													
Temperature	2	4.49×10^{-2}	2.24×10^{-2}	2.20	0.192	1063.5	531.7	2.33	0.179	7.09×10^{-3}	3.54×10^{-3}	18.23	0.003
Regression	1	2.89×10^{-2}	2.89×10^{-2}	2.84	0.143	498.8	498.8	2.18	0.190	0.94×10^{-3}	0.94×10^{-3}	4.83	0.070
Deviation	1	1.60×10^{-2}	1.60×10^{-2}	1.57	0.257	564.7	564.7	2.47	0.167	6.15×10^{-3}	6.15×10^{-3}	31.62	0.001
Replicate	6	6.11×10^{-2}	1.02×10^{-2}	15.47	<0.001	1370.4	228.4	17.56	<0.001	1.17×10^{-3}	0.19×10^{-3}	0.64	0.696
Error	891	0.59	6.58×10^{-4}			11592.9	13.0			0.27	0.30×10^{-3}		
(b) Males													
Temperature	2	3.73×10^{-2}	1.86×10^{-2}	1.46	0.304	789.7	394.9	1.94	0.223	8.77×10^{-3}	4.39×10^{-3}	6.79	0.029
Regression	1	1.29×10^{-2}	1.29×10^{-2}	1.01	0.353	238.6	238.6	1.17	0.320	4.62×10^{-3}	4.62×10^{-3}	7.14	0.037
Deviation	1	2.44×10^{-2}	2.44×10^{-2}	1.91	0.216	551.1	551.1	2.71	0.151	4.15×10^{-3}	4.15×10^{-3}	6.42	0.044
Replicate	6	7.67×10^{-2}	1.28×10^{-2}	19.78	<0.001	1220.7	203.4	20.12	<0.001	3.88×10^{-3}	0.65×10^{-3}	1.88	0.082
Error	1059	0.68	6.46×10^{-4}			10709.3	10.1			0.36	0.34×10^{-3}		

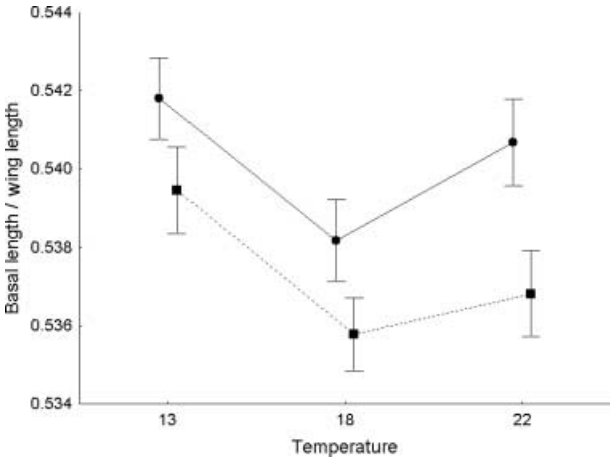

Fig. 2 Averages of the relative length (with 95% confidence intervals) of the basal portion of longitudinal vein IV (L1) to the total wing length (WL = L1 + L2) vs. experimental temperature for females (●) and males (■). All three replicated populations within each thermal regime were pooled because no statistically significant differences among replicates were detected in the two-way nested ANOVAS (see Table 2).

latitude) and in recently colonizing populations of North America (a negative correlation with latitude; Huey *et al.*, 2000).

Wing shape variability

The Procrustes two-level nested ANOVA for female wing shape variation led to the same previous conclusion regarding shape index L1/WL; namely, a significant and consistent (across replicated populations) shape variation was detected among thermal selection regimes but with a nonlinear trend for temperature (Table 3a. Notice that probabilities from the two-stage permutation test are higher than those from a 'true' *F*-statistics with the same degrees of freedom, which clearly indicates that the degrees of freedom in our Procrustes ANOVA are not independent and also suggests correlated landmark shifts.) However, no statistically significant differences were detected for males (Table 3b), which might suggest that they are lagging behind females. [Actually, when Procrustes distances were used as the dependent variable (which allows for global tests of shape differences), significant effects of thermal selection regimes were detected for both sexes (see below).] A differentiated pattern between sexes also emerged when variance components from the Procrustes ANOVAS were apportioned by landmarks. Thus, landmarks 1, 2 and 13 dominated for temperature effects in females but had relatively low amounts of variability in males. However, landmark 5 had the largest temperature effect in males (Fig. 3). To summarize, although the relative amounts of variation at each landmark vary markedly among the factors included in the two-way nested ANOVAS, temperature effects seem to be significant in both sexes for at least one landmark involved in the shape index L1/WL (as could be expected from the results in Table 2).

Principal component analyses were carried out for overall shape variation among individuals (within sexes) across the entire wing (i.e. from the covariance matrices of the coordinates of superimposed landmarks obtained from the 900 females and the 1068 males), as well as among thermal regimes (i.e. from the covariance matrices of the mean coordinates at each temperature). For individual variation the first two principal components explained at least 63 and 7% of the variability, respectively, and results were fairly consistent between sexes (Fig. 4). This high level of variability explained by a few PCs clearly suggests strong dependencies among landmarks and, hence, the isotropic model (which presumes that there is an equal amount of nondirectional variation

Table 3 Two-level nested analyses of variance for shape using Procrustes sums of squares as a measure of overall variation in shape. Statistical significance was computed after 10 000 random permutations (see text for details).

Source	d.f.	SS	MS	*F*	*P*
(a) Females					
Temperature	44	11.58×10^{-3}	2.63×10^{-4}	4.02	0.019
Regression	22	2.36×10^{-3}	1.07×10^{-4}	1.64	0.196
Deviation	22	9.22×10^{-3}	4.19×10^{-4}	6.40	0.007
Replicate	132	8.63×10^{-3}	0.65×10^{-4}	1.81	0.071
Error	19 602	696.81×10^{-3}	0.36×10^{-4}		
(b) Males					
Temperature	44	5.53×10^{-3}	1.26×10^{-4}	1.61	0.187
Regression	22	1.98×10^{-3}	0.90×10^{-4}	0.15	0.318
Deviation	22	3.55×10^{-3}	1.61×10^{-4}	2.07	0.113
Replicate	132	10.29×10^{-3}	0.78×10^{-4}	2.02	0.016
Error	23 298	899.41×10^{-3}	0.39×10^{-4}		

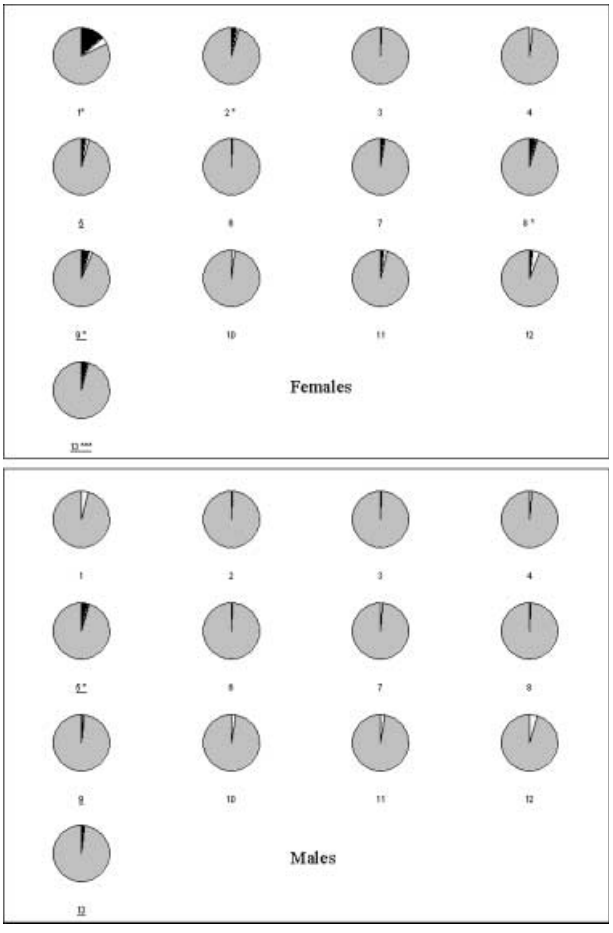

Fig. 3 Pie charts indicating the percentage of the variance for the effects of temperature (black), replicate (white) and error (grey) in the Procrustes ANOVAS on individual landmarks. Those landmarks used to estimate shape index (basal length/wing length) are underlined. Significance of temperature effect are indicated by asterisks (*$P < 0.05$; ***$P < 0.001$).

at each landmark) does not seem to hold (see Dryden & Mardia, 1998, p. 97). The effects of between-individual variation were distributed in a relatively even way among all landmarks. Outer landmarks tended to move in the direction of an enlarged wing aspect; namely, to decrease wing width relative to WL. However, the direction of PCs is arbitrary and all the movements can be simultaneously reversed by 180°. Therefore, the best interpretation is to describe overall shape variation along a wide–narrow direction. The PC2 mainly consisted of a widening (narrowing) of the posterior compartment of the wing (which includes those landmarks located below an imaginary line situated approximately along the fourth longitudinal vein; see García-Bellido & de Celis, 1992). The PC3 simultaneously involved two landmarks on the fourth longitudinal vein (5 and 13) shifting to reverse directions, resulting in an increase (decrease) of the length of the distal segment L2. Conventional morphometric methods performed on wing size measurements in samples from Europe and North America also detected an inverse relationship (accounting for 10% of the variance) between the proximal (L1) and distal (L2) portions of longitudinal vein IV, as well as between WL and width (Gilchrist *et al.*, 2001).

For temperature effects there are only two PCs, and the features of shape variation are graphically shown in Fig. 5. Some slight differences between sexes are now apparent (but recall that Procrustes ANOVAS did not detect statistically significant thermal effects on males; Table 3). The PC1 in females was connected to the large variability previously detected for landmark 1 (see Fig. 3), which moves towards landmark 9. However, several landmarks had relatively large PC1 coefficients in males. Landmarks 13 and 7, which define the position of the posterior cross vein, shift to the same direction in both sexes. Similar to PC3s for individual variation, landmarks 5 and 13 seem to move in opposite directions but these shifts were quite small in females. The PC2 was mainly involved with the shift of landmark 5 in males and females.

Rates of genetic divergence for wing shape

Rates of wing size evolution and divergence on a continental scale have been estimated to be very fast in *D. subobscura* (Huey *et al.*, 2000; Gilchrist *et al.*, 2001). We did not detect here any significant effect of thermal selection regimes on wing size, but it could be interesting to estimate the rates of divergence for wing shape in the experimental populations (Hendry & Kinnison, 1999 describe this as the synchronic method). To handle wing shape as a single metric we used Procrustes distances and calculated them as $\rho = 2 \sin^{-1}(d_p/2)$, where d_p is the square root of the sum of square differences between corresponding points (see Rohlf, 1999). This procedure obviously reduces the inherently multidimensional shape data to a single magnitude of shape differences,

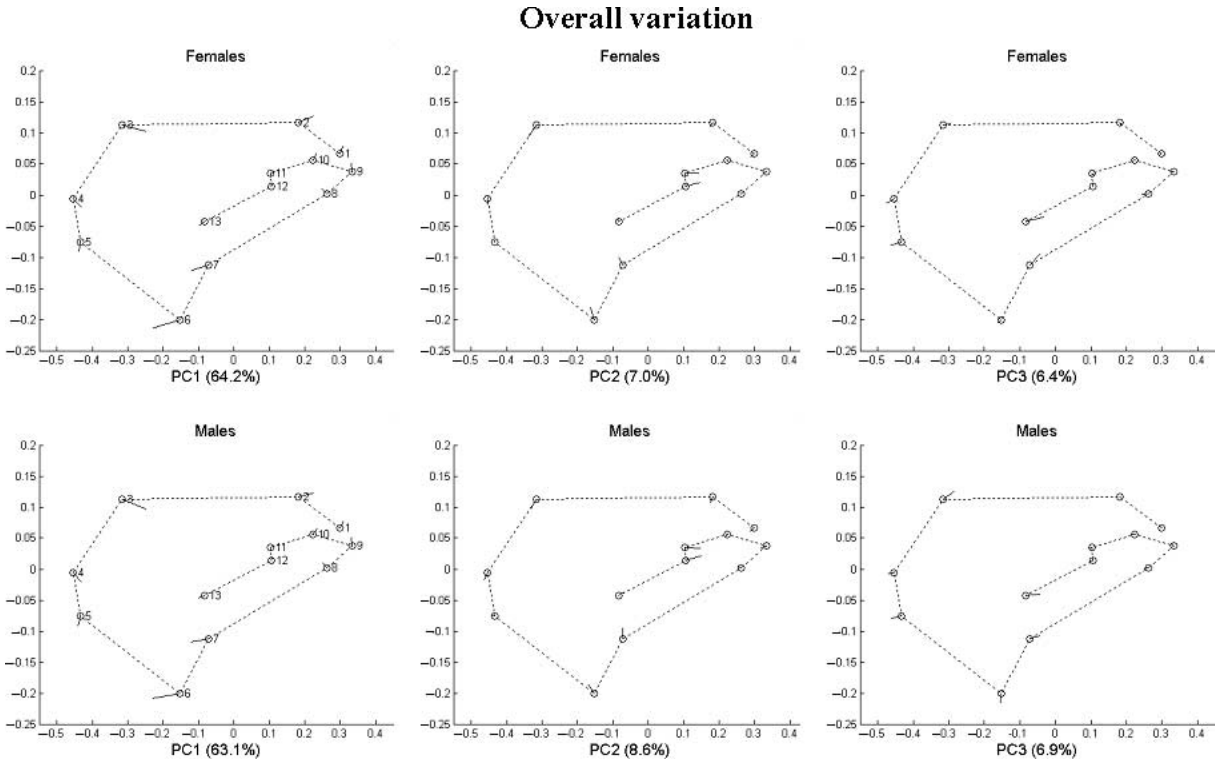

Fig. 4 Principal components of the covariance patterns in landmarks shifts due to among-individual variation for each sex. The PC coefficients are shown as a solid line originating at the mean location of the landmark (open circles) and ending at the location to which the landmark would move to +6 (PC1), +10 (PC2) and +12 (PC3) standard deviations (obviously an exaggeration of the variation in the dataset). The proportion of total variation accounted by each PC is given in brackets.

and ignores the direction of landmarks' shifts against each other (Goodall, 1991; Klingenberg, 2003; but see Monteiro *et al.*, 2003). However, the Procrustes distances are informative as summary statistics and can be used to investigate evolutionary rates of shape change.

Two-level nested ANOVAS applied to the Procrustes distances also rendered significant and consistent (across replicated populations) shape variation among thermal selection regimes for both sexes (females: $F_{44,132} = 6.97$, $P < 0.05$; males: $F_{44,132} = 7.51$, $P < 0.05$. Probabilities were computed after 10 000 random permutations as described in Materials and methods); and *post hoc* comparisons detected statistically significant differences when contrasting 18 °C vs. 13 °C or 22 °C in females, and 13 °C vs. 22 °C in males (analyses not shown). Incidentally, although qualitative conclusions obtained from shape index L1/WL (see Table 2 and above) and from Procrustes distances are the same, both variables are loosely correlated (females: Spearman $r_S = -0.020$, n.s.; males: $r_S = -0.068$, $P < 0.05$).

Divergence rates for wing shape were estimated to be 0.0105 haldanes (2.1×10^4 darwins; 13 °C vs. 18 °C) and 0.0106 haldanes (2.7×10^4 darwins; 18 °C vs. 22 °C) in females, and 0.0068 haldanes (1.7×10^4 darwins; 13 °C vs. 22 °C) in males. Our approach of comparing only those means that showed statistically significant differences in *post hoc* contrasts can be obviously criticized (see Hendry & Kinnison, 1999). However, the previous figures do suggest that rates of genetic divergence for wing shape can be as fast or even faster than those estimated for wing size (cf. with the values reported by Gilchrist *et al.*, 2001).

Relationship between chromosomal polymorphism and wing shape index

The frequencies of chromosomal gene arrangements in the original natural population at Puerto Montt, in the initial founding population, and in the thermal selection stocks after 1 year of divergence are shown in Table 4. Three-way log-linear analyses (including experimental temperature, replicate, and gene arrangement as the main effects) performed for each chromosome showed that the frequencies of some gene arrangements have already changed according to experimental temperature, but the three replicate populations within each temperature were homogeneous and could be lumped together (results not shown). Our aim here, however, is not to discuss those changes in gene arrangement frequencies but to relate the simultaneously analysed chromosomal

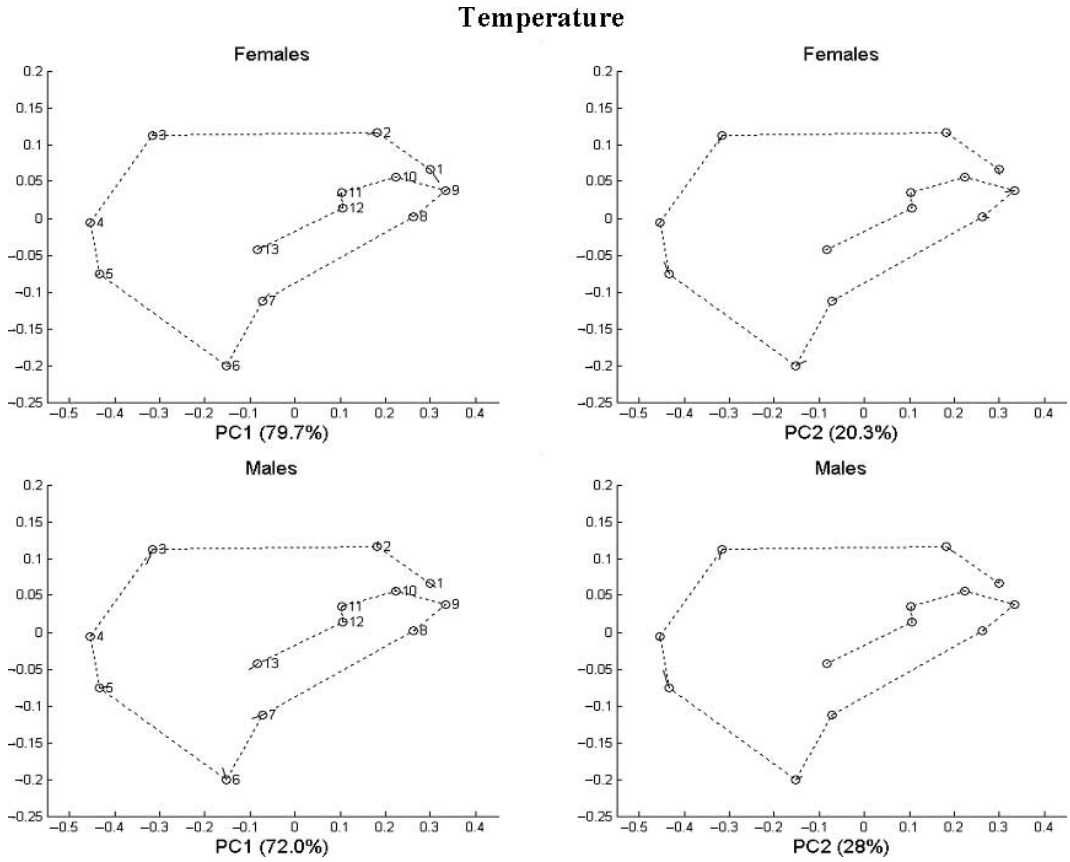

Fig. 5 Principal components of the covariance patterns in landmarks shifts due to among-temperature variation for each sex. The end points of the solid lines are at locations displaced +15 (PC1) and +30 (PC2) standard deviations from the mean configuration.

polymorphism and wing shape index in the males sampled from the thermal stocks. For this purpose we have used the 'standard dose' (i.e. the number of standard gene arrangements carried out by a male, which ranges from 0 to 5) as the relevant variable. The reason is that the various gene arrangements in Table 4 can be divided in two groups based on the correlation of gene arrangement frequencies and latitude: the 'cold-adapted' group comprised by A_{st}, J_{st}, U_{st}, E_{st} and O_{st}; and the 'warm-adapted' group involving A_2, J_1, U_{1+2}, U_{1+2+8}, $E_{1+2+9+3}$, $E_{1+2+9+12}$, O_{3+4} and O_{3+4+8} (arrangements E_{1+2+9}, O_{3+4+2} and O_{3+4+7} do not show a clear latitudinal pattern; see Menozzi & Krimbas, 1992). Because the frequencies of some standard gene arrangements were relatively low, we have grouped the low frequencies at the upper tail of the distribution and the relationship between shape index L1/WL and the standard dose is plotted in Fig. 6 for each experimental temperature. In all cases, the length of L1 relative to total WL sharply decreased with the standard dose [dependent variable $\log_e$(L1/WL); 13 °C: $\beta = -5.16 \times 10^{-3}$, $F_{1,328} = 20.11$, $P < 0.001$; 18 °C: $\beta = -3.85 \times 10^{-3}$, $F_{1,358} = 12.37$, $P < 0.001$; 22 °C: $\beta = -6.46 \times 10^{-3}$, $F_{1,324} = 26.23$, $P < 0.001$], and the regression coefficients were not statistically heterogeneous [$F_{2,1010} = 1.18$, n.s. Overall $\beta = -5.10 \times 10^{-3}$ (95% confidence limits: -6.42×10^{-3}, -3.78×10^{-3})]. It thus seems quite clear that polymorphic gene arrangements in *D. subobscura* have a consistent (across temperatures) biometrical effect on wing shape. The standard dose in the thermal stocks increased with increasing temperature, which could somewhat explain the pattern observed in Fig. 2 for males (in fact, the statistically significant effect for temperature in Table 2b disappears when the standard dose is introduced in the analysis as a covariate; results not shown). Interestingly, the decrease of wing shape index with latitude observed in North American colonizing populations (Huey *et al.*, 2000) fully agrees with the present data because the standard gene arrangements generally increase with latitude in those populations (Balanyà *et al.*, 2003). Actually, the increase of standard dose with latitude for eight North American populations covering a latitudinal range of about 13° and sampled in 1994 was $\beta = 0.072 \pm 0.010$. As formerly pointed out we did not aim here to discuss the changes in gene arrangement frequencies in the thermal selection stocks, but just

Table 4 Chromosomal polymorphism in the original natural population at Puerto Montt, in the founding population, and in the thermal selection stocks after 1 year.

			Experimental populations											
	Natural	Founding	13 °C (generation 9) (May 2002)				18 °C (generation 12) (May 2002)				22 °C (generation 15) (May 2002)			
Chromosome arrangement	population (November 1999)	population (May 2001)	R1	R2	R3	Total	R1	R2	R3	Total	R1	R2	R3	Total
A														
A_{st}	0.507	0.306	0.248	0.157	0.236	0.213	0.198	0.226	0.258	0.227	0.096	0.164	0.224	0.160
A_2	0.493	0.694	0.752	0.843	0.764	0.787	0.802	0.774	0.742	0.773	0.904	0.836	0.776	0.840
N	134	121	117	115	110	342	126	124	120	370	114	110	107	331
J														
J_{st}	0.304	0.281	0.145	0.118	0.089	0.118	0.108	0.114	0.194	0.138	0.190	0.188	0.189	0.189
J_1	0.696	0.719	0.855	0.882	0.911	0.882	0.892	0.886	0.806	0.862	0.810	0.813	0.811	0.811
N	135	121	117	119	112	348	139	132	134	405	116	112	111	339
U														
U_{st}	0.422	0.512	0.265	0.269	0.277	0.270	0.312	0.220	0.239	0.257	0.448	0.339	0.351	0.381
U_{1+2}	0.348	0.281	0.538	0.479	0.482	0.500	0.529	0.591	0.515	0.545	0.353	0.438	0.477	0.422
U_{1+2+8}	0.230	0.207	0.197	0.252	0.241	0.230	0.159	0.189	0.246	0.198	0.198	0.223	0.171	0.198
N	135	121	117	119	112	348	138	132	134	404	116	112	111	339
E														
E_{st}	0.593	0.612	0.530	0.462	0.491	0.494	0.755	0.659	0.709	0.709	0.828	0.786	0.865	0.826
E_{1+2}	0.037	–	–	–	–	–	–	–	–	–	–	–	–	–
E_{1+2+9}	0.059	0.074	0.034	0.067	0.027	0.043	0.036	0.098	0.022	0.052	0.112	0.045	0.045	0.068
$E_{1+2+9+3}$	0.148	0.231	0.214	0.168	0.161	0.181	0.101	0.076	0.179	0.119	0.043	0.116	0.063	0.074
$E_{1+2+9+12}$	0.163	0.083	0.222	0.303	0.321	0.282	0.108	0.167	0.090	0.121	0.017	0.054	0.027	0.032
N	135	121	117	119	112	348	139	132	134	405	116	112	111	339
O														
O_{st}	0.289	0.190	0.111	0.109	0.045	0.089	0.101	0.098	0.082	0.094	0.121	0.018	0.090	0.077
O_{3+4}	0.081	0.050	0.068	0.034	0.080	0.060	0.072	0.045	0.075	0.064	0.181	0.259	0.189	0.209
O_{3+4+2}	0.252	0.421	0.581	0.588	0.625	0.598	0.619	0.606	0.500	0.575	0.534	0.455	0.550	0.513
O_{3+4+7}	0.126	0.140	0.085	0.143	0.116	0.115	0.108	0.114	0.179	0.133	0.043	0.125	0.108	0.091
O_{3+4+8}	0.170	0.149	0.154	0.109	0.125	0.129	0.101	0.136	0.157	0.131	0.121	0.125	0.063	0.103
O_5	0.074	0.050	–	0.008	0.009	0.006	–	–	0.007	0.002	–	0.018	–	0.006
O_7	0.007	–	–	0.008	–	0.003	–	–	–	–	–	–	–	–
N	135	121	117	119	112	348	139	132	134	405	116	112	111	339

notice that the trend between the standard dose and temperature in these stocks after 1 year of divergence goes in the opposite direction to that expected from the latitudinal clines.

Discussion

Flies of both sexes that have diverged for 27 generations at most were found to be about the same size independently of thermal selection regime. The lack of divergence in wing size could probably have been expected from results in the first surveys conducted on *D. subobscura* flies from New World populations, which failed to show any latitudinal size clines 8 years after colonization (~40 generations; see Pegueroles *et al.*, 1995). Further samples from the laboratory populations collected after longer divergence times will allow us to test predictions based on the size clines. However, features of wing shape that differ between experimental treatments (and sexes) were found to be consistent within the three replicated populations, which clearly excludes any explanation of wing shape variation in *D. subobscura* grounded on drift around an optimum (Gilchrist *et al.*, 2001; see below).

Earlier experiments in *Drosophila* had also detected progressive differences in wing form between (unreplicated) stocks maintained at different temperatures (Cavicchi *et al.*, 1978). The changes, however, were not significant in any single dimension but were detected after a multivariate analysis. Further artificial selection experiments (Cavicchi *et al.*, 1981) led the authors to the conclusion that the individual wing dimensions are largely inseparable genetically. However, Weber (1990) has rightly argued that a shortcoming with the latter experiment is that the authors only selected on one wing dimension and in one direction, which is equivalent to directional selection for size (but not shape) because most of the genetic variance for size in any single dimension is simply variance for total size. When artificial selection is performed on angular offsets extensive localized effects are revealed, making it clear that the wing does not

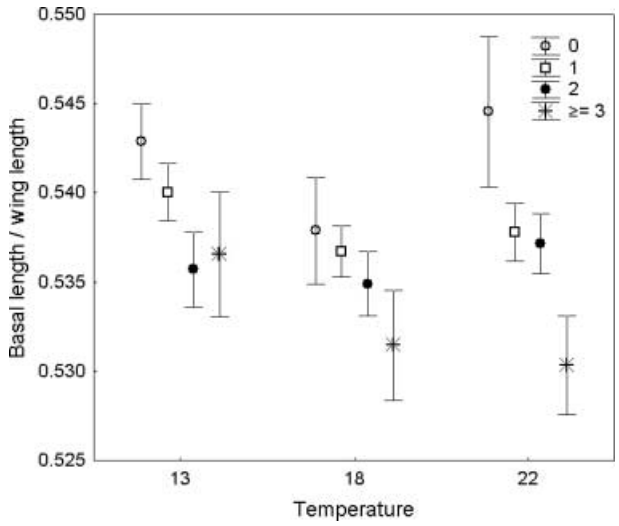

Fig. 6 Averages of the relative length (with 95% confidence intervals) of the basal portion of longitudinal vein IV (L1) to the total wing length (WL = L1 + L2) according to the dose (0, 1, 2 and ≥3) of standard gene arrangements carried by the sampled males at each experimental temperature. All three replicated populations within each thermal selection regime were pooled (see text for details).

evolve as a whole unit (Weber, 1990). Nevertheless, some contrasting patterns between overall wing variation (Fig. 4) and temperature effects (Figs 3 and 5) are apparent in our data set. In the first case the shifts of landmarks in PC1 did not occur in isolation, but also included most landmarks. Temperature has more localized effects in the shifts of landmarks. Similarly to the finding by Klingenberg & Zaklan (2000) in *D. melanogaster*, the shifts of the anterior (PC2 in Fig. 4) and posterior (PC3) cross veins appear to be rather independent of each other. These authors discuss similar displacements detected in both intra- and interspecific studies of wild-type flies, as well as in a number of mutant stocks.

In contrast to what has been uncovered for wing size, there are no consistent patterns between latitude and wing shape in *Drosophila*. Shape index L1/WL in *D. subobscura* increases with latitude in Europe and decreases in North America (Huey *et al.*, 2000), but shows no linear trend in South America (G. W. Gilchrist, pers. comm., 2002). In *D. melanogaster* Gilchrist *et al.* (2000) found that the main shape canonical variate also varied significantly between continents. Hoffmann & Shirriffs (2002) found a nonlinear trend between latitude and the first shape canonical variate in *D. serrata* flies from Australia, although there was a linear change in wing aspect (the ratio of WL to wing width). Parenthetically, the shape changes brought about by thermal selection regimes in our *D. subobscura* stocks were not associated with changes in wing-aspect ratio [estimated as (wing length)^2/wing area (see Azevedo *et al.*, 1998). Females: $F_{2,6} = 1.31$, n.s.; males: $F_{2,6} = 0.84$, n.s.]. Overall the results of these studies, as far as can be judged from the published reports on traditional and geometric morphometrics, quantitative genetics and QTL analyses of *Drosophila* wing shape (Weber, 1990, 1992; Bitner-Mathé & Klaczko, 1999a,b; Weber *et al.*, 1999, 2001; Birdsall *et al.*, 2000; Klingenberg & Zaklan, 2000; Zimmerman *et al.*, 2000; Gilchrist & Partridge, 2001), suggest that size and shape have different genetic properties and do not respond to the same environmental factors. In summary, the steady geographical clines for size across continents and *Drosophila* species, with wing size increasing with latitude largely independently of the underlying details in the genetic architecture (Gilchrist & Partridge, 1999), strongly support the hypothesis that body size (or the correlated trait growth rate) is the target of selection but there is a localized and richly structured variation in wing shape.

As wing shape seems to be strongly resistant to environmental influences (Weber, 1990; Birdsall *et al.*, 2000) and there is little compelling evidence indicating that natural wing shape changes are adaptive in *Drosophila*, it would be premature to attempt to explain the high rates of genetic divergence in our thermal selection stocks in functional terms [notwithstanding Imasheva *et al.*'s (1995) conclusions]. Wing shape is indeed remarkably constant within inbred lines (Birdsall *et al.*, 2000); however, it does not seem to be strongly canalized against genetic change and responds to divergent selection in the same way as most quantitative traits, with some genes causing small and localized effects (Weber, 1990, 1992). Many genes with small additive effects on wing shape are dispersed along the *Drosophila* genome (Weber *et al.*, 1999, 2001; Zimmerman *et al.*, 2000). This suggests plentiful chances for gene-inversion linkage disequilibria in the inversion-rich species *D. subobscura*, particularly in samples derived from New World colonizing populations (actually, linkage disequilibria between microsatellite loci are almost absent in European populations but are detectable in New World populations; M. Pascual, pers. comm., 2002). The thermal selection stocks have already diverged for various gene arrangements with no differences between replicated populations (see above), and a strong relationship between shape variables and polymorphic inversions (i.e. shape index L1/WL sharply decreased at all experimental temperatures as the dose of standard chromosomal arrangements increased) was uncovered. In addition, our findings strongly suggest that the latitudinal clines for chromosomal gene arrangements may account for the wing shape cline in North America colonizing populations (Huey *et al.*, 2000). Even more, we could hypothesize that the shape cline in North America predated the size cline because of the quite different paces between inversion and size clines (see above). To test this hypothesis we have reanalysed the data reported in Pegueroles *et al.* (1995) for six North American populations sampled by A. Prevosti and M. Monclús in July 1986 (4 years after the

initial colonization in North America) before the size cline built up. As predicted, for both females [dependent variable $\log_e$(L1/WL); $\beta = -0.92 \pm 0.27 \times 10^{-3}$] and males ($\beta = -0.49 \pm 0.26 \times 10^{-3}$) the length of L1 relative to total WL decreased with latitude, but the colonizing males were lagging behind females and this tendency is still apparent in more recent samples (see Gilchrist *et al.*, 2001). In summary, our results cast strong doubts on the supposed 'unpredictability' of the geographical cline in *D. subobscura* North American colonizing populations (Huey *et al.*, 2000).

The use of geometric morphometrics together with quantitative genetic studies is an ambitious project and requires large data sets (see Klingenberg & Leamy, 2001; Klingenberg, 2003). We are currently analysing male's multivariate wing shape tangent space in relation to inversions, in addition to carrying out quantitative genetic analyses in a set of isochromosomal lines fixed for different O chromosome arrangements. In the meantime, the most parsimonious explanation for our present results is that changes in gene arrangement frequencies as a response to temperature likely underlie the correlated changes in wing shape because of gene-inversion linkage disequilibria.

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Temperature-Related Genetic Changes in Laboratory Populations of *Drosophila subobscura*: Evidence against Simple Climatic-Based Explanations for Latitudinal Clines

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abstract: Parallel latitudinal clines to the long-standing ones in the original Palearctic populations have independently evolved at different rates for chromosomal polymorphism and body size in South and North American populations of *Drosophila subobscura* since colonization around 25 years ago. This strongly suggests that (micro) evolutionary changes are largely predictable, but the underlying mechanisms are unknown. The putative role of temperature per se was investigated by using three sets of populations at each of three temperatures (13°, 18°, and 22°C) spanning much of the tolerable range for this species. We found a lower chromosomal diversity at the warmest temperature; a quick and consistent shift in gene arrangement frequencies in response to temperature; an evolutionary decrease in wing size, mediated by both cell area and cell number, at 18°C; no relationship between wing size and those inversions involved in latitudinal clines; and a shortening of the basal length of longitudinal vein IV relative to its total length with increasing standard dose. The trends for chromosomal polymorphism and body size were generally inconsistent from simple climatic-based explanations of worldwide latitudinal patterns. The findings are discussed in the light of available information on *D. subobscura* and results from earlier thermal selection experiments with various *Drosophila* species.

Keywords: chromosome polymorphism, clinal variation, *Drosophila subobscura*, thermal evolution, wing size, wing shape.

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Latitudinal and altitudinal clines are prominent indicators of natural selection at work over large geographic scales. Some classical examples from *Drosophila* species are gradients in chromosomal inversion frequencies (e.g., Dubinin and Tiniakov 1946; Stalker 1976; Mettler et al. 1977; Levitan 1978; Knibb et al. 1981; Menozzi and Krimbas 1992; van't Land et al. 2000; Schaeffer et al. 2003; see Krimbas and Powel 1992 for review) and geographical clines in body size with genetically larger individuals derived from higher latitudes (Stalker and Carson 1947; Prevosti 1955; Misra and Reeve 1964; David et al. 1977; Coyne and Beecham 1987; Pegueroles et al. 1995; James et al. 1997; van't Land et al. 1999; Huey et al. 2000). The most convincing evidence that such clines are adaptive probably comes from the temperate (i.e., cold tolerant) species *Drosophila subobscura.*

Formerly restricted to the Palearctic region, these flies were discovered in South America at the locality of Puerto Montt (Chile) in February 1978 (Brncic and Budnik 1980) and explosively colonized a broad latitudinal range (Brncic et al. 1981; Prevosti et al. 1985). Also remarkable was the discovery in 1982 of *D. subobscura* in North America near Port Townsend, Washington (Beckenbach and Prevosti 1986), because those two colonization events could now

be considered a large-scale natural experiment with two replicates (i.e., "a grand experiment in evolution"; Ayala et al. 1989). The regular tracking of the inversion polymorphism and body dimensions ever since the colonization of the Americas by *D. subobscura* has resulted in a number of evolutionarily important findings (summarized in Gilchrist et al. 2001, 2004; Serra 2002; Balanyà et al. 2003). First, almost all inversions that occur in New World populations have shifted in frequency as a response to environmental gradients, and latitudinal clines were soon detected. Second, those inversions show the same correlation sign of frequency with latitude as in the original Old World populations. Third, inversion clines have not continued to converge on the native Old World baseline. Fourth, after approximately 20 years from introducing the species into South and North America, geographical clines have also evolved for wing length that are morphologically equivalent to the original cline in the Old World; however, fifth and finally, the underlying details by which the body size clines were achieved—either the cellular basis of different size clines (Calboli et al. 2003*a*) or the particular wing segment causing the cline (Huey et al. 2000)—seem to be contingent (but see Santos et al. 2004). Therefore, the conclusion that the inversion polymorphism and body size of *D. subobscura* are selectively responding to worldwide parallel environmental conditions is quite compelling.

The repeatability of body size clines across continents and *Drosophila* species, together with laboratory experiments on thermal evolution where adaptation to lower temperature consistently resulted in increased body size (Anderson 1966; Powell 1974; Cavicchi et al. 1989; Partridge et al. 1994*a*), has led most researches to conclude that geographical clines in *Drosophila* body size are indeed the result of adaptation to different temperatures (albeit a thorough understanding of temperature-dependent body size variation in ectotherms is still lacking). The snag is, however, that unintended selection due to failures in controlling conditions in life cycle stages that are not directly targeted by the selection procedures can spoil the interpretation of results (see Rose et al. 1996). In the aforementioned thermal selection experiments, the most commonly neglected variable was larval culture density across temperatures. For instance, in *Drosophila melanogaster*, it is well known that populations stabilize at lower densities when maintained at lower temperatures, and, hence, crowding conditions differ between thermal selection stocks (L. Partridge, personal communication, 1998). Spurious temperature-related adjustments of adult body size may then result because physiological adaptations to temperature were not independent of adaptations to different levels of larval crowding, which, in turn, involve changes in larval behavior and physiology that may impinge on other phases of the life cycle (see, e.g., Joshi and Mueller 1996; Santos 1996; Santos et al. 1997; Houle and Rowe 2003). In summary, most laboratory thermal selection experiments carried out so far have the drawback that different levels of harmful larval waste products (Borash et al. 1998, 2000) and food shortage due to various crowding conditions co-varied with selection pressures in shaping larval resource processing at various temperatures (Bochdanovits and de Jong 2003*a*, 2003*b*).

With the aim of studying the short- and long-term outcomes of thermal selection on the chromosomal inversion polymorphism and wing size and shape of *D. subobscura*, we have developed a set of three replicated populations kept at three experimental temperatures under controlled larval densities (see Santos et al. 2004): cold (13°C), optimum (18°C), and warm (22°C). These populations have been sampled twice: after 1 and 2 years since foundation when they had already diverged for 27 and 51 generations at most, respectively. The results certainly show that the chromosomal inversion polymorphism has quickly and consistently responded to thermal regime, but the trends were generally not in harmony with predictions based on latitudinal clines or continent-wide shifts putatively caused by global warming (e.g., Solé et al. 2002). Contrary to what had been observed in the first sample, temperature-related genetic differences in wing size were detected after 2 years, but warm-adapted populations were not smaller than their cold-adapted counterparts and had about the same wing cell area. Finally, we provide convincing evidence that New World latitudinal clines for polymorphic inversions and body size in *D. subobscura* are clearly uncoupled. We discuss the results in the light of available information on the inversion polymorphism of *D. subobscura* and also challenge the idea that *Drosophila* body size is the target of thermal selection.

Material and Methods

Experimental Populations and Sampling Protocol

All nine populations used here were initiated from an ancestral population of *Drosophila subobscura* derived from a large outbred stock collected in November 1999 at the estimated Chilean epicenter of the original New World invasion (Puerto Montt, Chile, 41°28′S; see Brncic and Budnik 1980). From that ancestral population, three sets (13°, 18°, and 22°C) of three replicate populations each (R1, R2, and R3) were set up in May 2001. These thermal selection stocks are continuously kept on a discrete generation, controlled larval crowded regime on a 12L : 12D period. Prior to initiating a new generation, eclosed adults from the bottles are dumped into a Plexiglas cage (27 cm × 21 cm × 16 cm) and supplied with liberal amounts

of food before egg collections. The number of breeding adults per population is typically well over 1,500 flies. Complete details of the derivation and maintenance of these populations have previously been described (Santos et al. 2004).

Simultaneously to the establishment of the thermal selection stocks, a large sample of eggs were collected to estimate the frequencies of the chromosomal arrangements in the founding population. Samples from all nine populations were next taken after 1 (May 2002: nine generations at 13°C, 12 at 18°C, and 15 at 22°C) and 2 years (April 2003: 16 generations at 13°C, 22 at 18°C, and 29 at 22°C) of thermal selection (see Santos et al. 2004 for experimental details). In order to control for the possibility of nongenetic parental effects on offspring size (Crill et al. 1996), the parents of sampled flies had also been reared at the same temperature of 18°C. Experimental flies were separated by sex; females were stored in Eppendorf tubes with a 3 : 1 mixture of alcohol and glycerol at 4°C, and males (125–150 males per population randomly chosen from the 12 replicated bottles) were individually crossed in vials (2 cm × 8 cm containing 6 mL of food) to three to four virgin females from the *ch-cu* marker strain in order to estimate chromosome arrangement frequencies (see below). After approximately 9 days, the males were removed from the vials and individually fixed in a 3 : 1 mixture of alcohol and glycerol at 4°C. All fly handling was done at room temperature using CO_2 anesthesia on flies not less than 6 h after eclosion.

Chromosomal Inversions

The karyotype of *D. subobscura* consists of five acrocentric chromosomes and a dot chromosome. Following Mainx et al. (1953), the large chromosomes in this species are traditionally named as A (=X, the sex chromosome), J (=chromosomal element D of Mueller/Sturtevant/Novitski and homologous to arm 3L in *Drosophila melanogaster*; see Powell 1997, p. 307), U (=chromosomal element B and homologous to arm 2L), E (=chromosomal element C and homologous to arm 2R), and O (=chromosomal element E and homologous to arm 3R). In colonizing populations of the New World, a total of 18 chromosomal arrangements (see Balanyà et al. 2003) have been found out of approximately 92 (produced from 66 inversions) recorded in the Palearctic region (Krimbas 1992, 1993; Menozzi and Krimbas 1992). All those 18 arrangements were present in the sample from Puerto Montt used to derive the thermal selection stocks: namely, A_{st}, A_2, J_{st}, J_1, U_{st}, U_{1+2}, U_{1+2+8}, E_{st}, E_{1+2}, E_{1+2+9}, $E_{1+2+9+3}$, $E_{1+2+9+12}$, O_{st}, O_{3+4}, O_{3+4+2}, O_{3+4+7}, O_{3+4+8}, and O_5 (for complete details of the naturally occurring inversions in *D. subobscura*, see Krimbas and Loukas 1980; Krimbas 1993). In addition, arrangement O_7 was also present at Puerto Montt but at a very low frequency (0.7%; see table A1 in the online edition of the *American Naturalist*). This arrangement, which is also found periodically in the Old World, is probably the result of a recombination event in the O_{3+4+7}/O_{st} heterokaryotype.

In *D. subobscura*, it is difficult to recognize the gene arrangements in all possible homozygous/heterozygous combinations. Therefore, males were individually crossed to three or four virgin females from the *ch-cu* marker strain in order to estimate chromosome arrangement frequencies. This strain is homozygous for the morphological recessive markers on the O chromosome cherry eyes (*ch*) and curled wings (*cu*; Koske and Maynard Smith 1954), and its genetic background is highly homogeneous and fixed for the standard gene arrangements in all major acrocentric chromosomes but chromosome O, where it is fixed for gene arrangement O_{3+4} (Lankinen and Pinsker 1977). Whenever feasible, one F_1 female third-instar larva derived from each cross with the homozygous *ch-cu* stock was examined for its inversion loops in polytene chromosomes to determine the gene arrangements of one set of the chromosomes from the wild-type male. Sample sizes were always larger than 100 gametes per population (table A1).

Wing Measurements

Wing size and shape of both sexes from each experimental population were measured as previously described (Santos et al. 2004). Briefly, wings were removed from each fly and fixed in DPX under coverslips on microscope slides. A total of ~100 females per population selected haphazardly from the 12 replicated bottles (see above), as well as the majority of males crossed to the *ch-cu* marker strain for chromosomal study, were used for measurements. Bitmap images of the wings were captured with a Sony CCD-Iris video camera connected to a PC with MGI VideoWave software and mounted on a Zeiss Axioskop compound microscope, using a 2.5× objective. The images were stored on a Dell Workstation PWS350 computer and then used to record the *x* and *y* coordinates of 13 morphological landmarks (fig. A1 in the online edition of the *American Naturalist*), using the Scion Image for Windows software (http://www.scioncorp.com). All the data used here are from the left wings (which happen to be slightly bigger than the right wings; Iriarte et al. 2003), and all wings have been measured by M. Santos. Using the original landmark coordinates, wing size was estimated in two different ways: as centroid size (CS) and as wing length (WL). The CS is defined as the square root of the sum of squared distances of a set of landmarks from their centroid or, equivalently, the square root of the sum of the variances

of the landmarks about that centroid in x and y directions (Slice et al. 1996). Following Robertson and Reeve (1952) and Prevosti (1955), WL was estimated as the combined lengths of the basal (labeled as L1) and distal (labeled as L2) segments of longitudinal vein IV (fig. A1). Wing shape was also estimated as the ratio of the basal length of longitudinal vein IV to the total wing length (i.e., L1/WL). The reason is that these linear measurements have been previously used to study size and shape clines in *D. subobscura* (e.g., Pegueroles et al. 1995; Huey et al. 2000; Gilchrist et al. 2001, 2004).

To estimate the cellular components of body size differentiation, a random sample of the slides including 50 females and 50 males per population already used for wing measurements was sent to Bologna. An image of the left wing was taken by V. Trotta at 40 × 10 magnification, and a sampling square of 11.55×10^{-3} mm^2 was selected in the area of the wing proximal to the posterior crossvein. Trichome counting followed a standard protocol; namely, the sampling area was visually inspected and the trichomes whose roots were within the selected square were marked with a black dot. Further manipulation provided a final image showing only the dots, which were counted using the ImageJ 1.31 software (http://rsb.info.nih.gov/ij/). Cell area was then estimated as 11.55×10^{-3} mm^2/dot number. Because cell area is variable across the wing blade, a total cell number index was estimated as $(L1 + L2)^2$ (mm^2)/cell area (mm^2).

Statistical Methods

Chromosomal Diversity. Chromosomal diversity was computed in three ways: as the number of segregating gene arrangements n_1; as the expected heterozygosity $H_E = 1 - \sum_{i=1}^{k} p_i^2$, where p_i is the relative frequency of gene arrangement i; and as $1 - \text{IFR} = \sum_{i,j=1, i\neq j}^{k} p_i p_j (1 - l_{ij})$, where IFR is the index of free recombination (Carson 1955) and l_{ij} is the proportion of euchromatin that freely recombines between gene arrangements i, j on the same chromosome. This index, which is claimed to be less biased than the mean number of inversions for which an individual is heterozygous (Krimbas and Loukas 1980; Krimbas 1993; Rodríguez-Trelles and Rodríguez 1998), is basically a measure of heterozygosity weighted by the relative length of the inversion, and a drop in $1 - \text{IFR}$ means that the population is becoming less heterogeneous for chromosomal arrangements. To compute the index, each heterokaryotype class was considered in turn, and the amount of euchromatin that is involved in inversion loops was obtained from the reference tables that measure the relative lengths of the inverted regions (Krimbas and Loukas 1979). For the total set of chromosomes, $1 - \text{IFR}$ was estimated as the weighted averages also taking into account the relative contents of total euchromatin in each chromosome element (see Wasserman 1982).

Inversion Polymorphism. The frequencies of chromosomal gene arrangements in the original natural population at Puerto Montt, in the initial founding population, and in the thermal selection stocks after 1 and 2 years of divergence are given in table A1. Data for the first year (May 2002) have been previously published but with the only purpose of relating male wing shape index L1/WL to the standard dose (i.e., the number of standard gene arrangements carried out by a male; see Santos et al. 2004) and are also included in table A1 for completeness.

The unit of analysis here is the population, and the three replicated populations (R1, R2, and R3) of each thermal selection stock were treated as a random factor nested within experimental temperature (13°, 18°, and 22°C), which was a fixed effect. This nested design somewhat precludes the use of three-way log-linear analyses (including experimental temperature, replicate, and gene arrangement as the main effects) to investigate variation in chromosomal arrangement frequencies because the replicated populations provide the appropriate error term to test for temperature effects (see Sokal and Rohlf 1995).

Since the various gene arrangements can be divided in two groups based on the correlation of gene arrangement frequencies and latitude, with A_{st}, J_{st}, U_{st}, E_{st}, and O_{st} included in the "cold-adapted" group (see Menozzi and Krimbas 1992; Balanyà et al. 2003), we can test the prediction that standard chromosomes are expected to decrease in frequency from 13°C to 22°C in the thermal selection stocks. The indicator variable x_{ij}, defined as $x_{ij} = 1$ if chromosome arrangement j in male i was standard and $x_{ij} = 0$ otherwise, was used in the analyses. Thus, the amount of variation for temperature effects in these analyses is the same as that obtained when using the standard gene arrangement frequency for a given chromosome weighted by the corresponding sample sizes, but we can further test for variation between replicated populations. In addition, the two degrees of freedom for temperature were partitioned into linear (regression) and nonlinear (deviation) effects. Permutation tests were used to assess whether the arrangement frequencies show significant variation in relation to temperature. For the two-level nested ANOVA model, randomization is a two-stage process: random permutations among replicate within temperature for the replicate in temperature F statistics, and random permutations among replicate and selection temperature for the among selection temperature F statistics. Each test used 10,000 random permutations of the observations.

Furthermore, year was introduced as a fixed effect in the previous two-level nested ANOVA to evaluate whether the slopes of inversion frequencies on experimental tem-

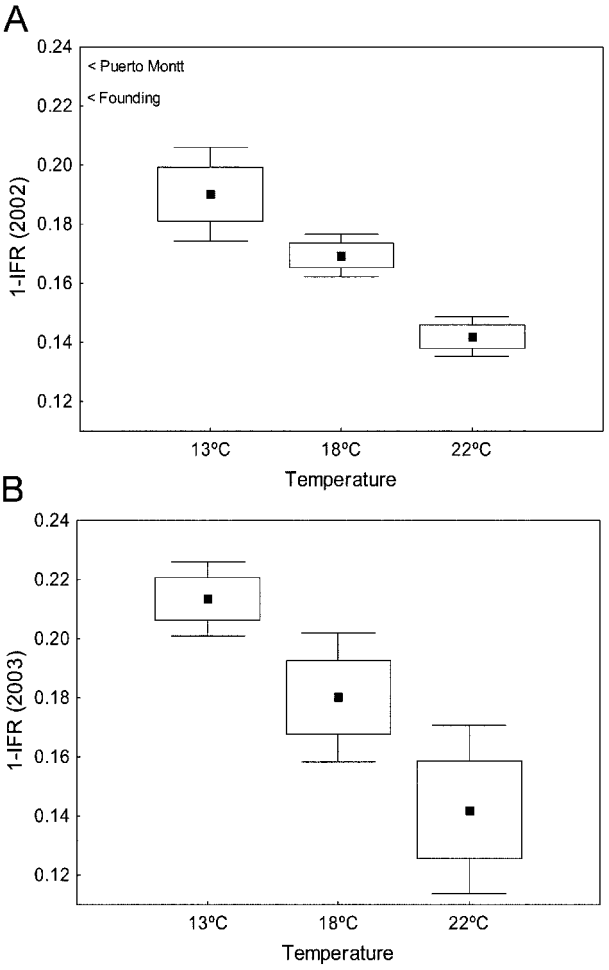


Figure 1: Boxplots showing the average chromosomal diversity (*boxes*: ± SE; *whiskers*: ± SD) for the total set of chromosomes of *Drosophila subobscura* in the thermal selection stocks. Chromosomal diversity was estimated here as 1 − IFR, where IFR is the index of free recombination (the relative contents of total euchromatin in each chromosome element were taken into account). The 1 − IFR values from the original population at Puerto Montt and from the founding population are also pointed in *A*.

perature were similar in the two samples. The interaction between temperature and year should be significant whenever slopes had changed in steepness. In the two-way ANOVA, randomization is a three-stage process (Good 1994; Edgington 1995), and the temperature × year interaction effect was tested from random permutations across temperatures and years after subtracting the deviations due to both main factors and adding the grand mean (see Sokal and Rohlf 1995). As before, each test used 10,000 random permutations.

Wing Variables. The wing traits measured (WL, CS, cell size, and cell number index $[L1 + L2]^2$/cell area) were analyzed separately (but see below). For each trait we used a standard linear model with replicated populations nested within experimental temperature, which was considered as a fixed effect. In addition, we tested the suggestion that *D. subobscura* wing size (WL) increases with the standard gene arrangement dose (Prevosti 1967; Orengo and Prevosti 2002), as well as our previous finding that polymorphic inversions have a biometric effect on the wing shape index L1/WL (Santos et al. 2004).

The computer programs used for statistical data analyses were MATLAB (MathWorks 2002) together with the collection of tools supplied by the Statistics Toolbox (MathWorks 2000). Results based on parametric ANOVAs were checked with the statistical software packages STATISTICA V.6 (StatSoft 2003) and SPSS (SPSS 2001).

Results

Chromosomal Diversity

A potentially important problem in our thermal selection stocks could be the loss of some or many of the naturally segregating polymorphic inversions in *Drosophila subobscura* in the relatively constant laboratory environment—as seems to happen with *Drosophila melanogaster* (e.g., Inoue 1979; Inoue et al. 1984)—for reasons unrelated to temperature adaptation. We have, therefore, first estimated the chromosomal diversity in the original population at Puerto Montt, in the founding population, and in the thermal stocks after 1 and 2 years since their establishment.

As formerly indicated, all $n_I = 18$ arrangements present in the New World were found at Puerto Montt, as well as the recombination-derived O_7, which was also sporadically uncovered in the experimental populations (see table A1). Gene arrangement E_{1+2}, which had a frequency of 3.7% at Puerto Montt and is not involved in the New World latitudinal clines (Balanyà et al. 2003), was likely lost in the founding population because it has not reappeared in any of the 2,295 slides scrutinized so far for chromosome E. All other gene arrangements (i.e., $n_I = 17$) are still segregating in the thermal stocks at various frequencies. Even the allelic lethal-carrying O_5 (Mestres and Serra 1995; Mestres et al. 2001; Iriarte et al. 2003), initially at a frequency of 5%, was recorded five times in 2002 (0.5%) and four times in 2003 (0.4%; table A1).

Figure 1 plots the chromosomal diversities (estimated as 1 − IFR) from the time of sampling at Puerto Montt in November 1999. Average chromosomal diversity at 13°C (0.190 in May 2002; 0.214 in April 2003) has remained close to the initial level in the founding population (0.225). However, it is quite obvious that there was a decreasing trend of chromosomal diversity with increasing tem-

perature. The Scheirer-Ray-Hare two-way nonparametric ANOVA (Sokal and Rohlf 1995, pp. 445–447) rendered a statistically significant temperature effect ($H = 6.37$, $P = .041$), but not year ($H = 0.56$, $P > .05$) or temperature × year interaction ($H = 0.03$, $P > .05$) effects.

Variation in Chromosome Arrangement Frequencies

Average frequencies for the standard chromosome arrangements in the thermal stocks are plotted in figure 2. Temperature effects were statistically significant for A_{st} and, most remarkably, E_{st} (table A2 in the online edition of the *American Naturalist*). Arrangement A_{st} showed a decline in frequency with increasing temperature, mainly due to the sharp drop at 22°C after 29 generations (fig. 2). On the other hand, the initial frequency of E_{st} dropped from 61.2% to 49.4% after nine generations at 13°C ($\Delta p = -0.0130$ per generation) and rose up to 82.6% after 15 generations at 22°C ($\Delta p = 0.0143$ per generation). The slope of the linear regression from the April 2003 sample was about the same as that in May 2002 (table A2); however, the baseline frequency (13°C) of E_{st} has further decreased at a similar rate from 49.4% in May 2002 to 40.2% in April 2003 ($\Delta p = -0.0132$ per generation). Conversely, E_{st} has remained at about the same frequency at 22°C (82.6% in May 2002 vs. 79.8% in April 2003). After an initial increase to 70.9% in May 2002, the frequency of E_{st} at 18°C had dropped in April 2003 (60.1%) to approximately the same level as that estimated in the founding population. All those figures suggest that the temperature-related shifts in E_{st} frequency mainly occur within the first ~15 generations. Thus, recall that in May 2002 the stocks at 22°C had already diverged for 15 generations since foundation, and in April 2003, those at 13°C had diverged for 16 generations. The per generation shift in frequency has been about the same but in reverse direction at the two extreme temperatures, so that their midpoint after 15–16 generations was exactly equal to the frequency of E_{st} in the founding population. The remaining standard gene arrangements for autosomes J, U, and O showed a nearly flat relationship with temperature, with the only exception perhaps being arrangement O_{st}, whose frequency has also dropped below 5% at 22°C (fig. 2).

Obviously, inversions A_2 and J_1 show patterns that are just the opposite of those observed for A_{st} and J_{st}. For the remaining three chromosomes, the situation is more complex. Aside from "random" fluctuations among replicated populations and/or sampling years, none of the three gene arrangements on chromosome U showed any trend with experimental temperature. Actually, after grouping all laboratory populations, their frequencies in April 2003 (i.e., $U_{st} = 36.4\%$, $U_{1+2} = 35.3\%$, and $U_{1+2+8} = 28.4\%$) were quite similar to those from the natural population at Puerto Montt ($U_{st} = 42.2\%$, $U_{1+2} = 34.8\%$, and $U_{1+2+8} = 23.0\%$). For chromosome E, a flat relationship with temperature was observed for arrangement E_{1+2+9}, whereas arrangements $E_{1+2+9+3}$ (May 2002: 18.1% at 13°C, 11.9% at 18°C, and 7.4% at 22°C; April 2003: 26.3% at 13°C, 18.6% at 18°C, and 10.8% at 22°C) and $E_{1+2+9+12}$ (May 2002: 28.2% at 13°C, 12.9% at 18°C, and 3.2% at 22°C; April 2003: 26.6% at 13°C, 11.9% at 18°C, and 3.5% at 22°C) showed a quite sharp and statistically significant linear decrease in frequency with increasing laboratory temperature. Finally, for chromosome O, arrangement O_{3+4} dramatically increased in frequency from 5% in the founding population to 21% at 22°C in May 2002. Its frequency in April 2003 was still significantly higher at 22°C (12.8%) than at the lower temperatures, where it remained at approximately the same initial frequency (~6%). Arrangements O_{3+4+2} and O_{3+4+7} showed opposite patterns in April 2003: the former significantly increased in frequency, and the latter significantly decreased in frequency at 22°C (table A1). In addition, a conspicuous trend for O_{3+4+2} has been a general increase in frequency since foundation (from 42.1% to 61.4% in April 2003; table A1). On the other hand, arrangement O_{3+4+8} did not show any trend with experimental temperature.

Thermal Evolution of Wing Traits

Wing Size. In contrast with the results after 1 year (Santos et al. 2004), two-level nested ANOVAs of wing size in the samples from April 2003 have already revealed statistically significant differences among thermal selection regimes for both females (WL as $\log_e[L1 + L2]$: $F = 19.64$, df = 2, 6, $P = .002$; CS [in pixels]: $F = 23.33$, df = 2, 6, $P = .001$) and males (WL: $F = 15.45$, df = 2, 6, $P = .004$; CS: $F = 20.09$, df = 2, 6, $P = .002$). Most striking was the pattern observed between body size and thermal selection regime (fig. 3; only the plots for WL are shown since there was a very high correlation between WL and CS: females $r = 0.981$, $P < .001$; males $r = 0.980$, $P < .001$). Thus, average WL (and CS) was about the same at the two extreme temperatures for both females (Scheffé post hoc tests using the "replicates" mean square as the error term: 13°C [2.7462 mm] vs. 22°C [2.7352], $P = .646$) and males (13°C [2.4717 mm] vs. 22°C [2.4548], $P = .462$) but showed a comparative and statistically significant reduction of ~2% at the optimum temperature of 18°C.

The between-year contrasts for WL should obviously be taken with great caution because of potential differences in the rearing conditions (though exactly the same experimental protocol and incubator shelf at 18° ± 0.1°C were used in both samples), but from figure 3 it seems reasonable to conclude that average wing size across rep-

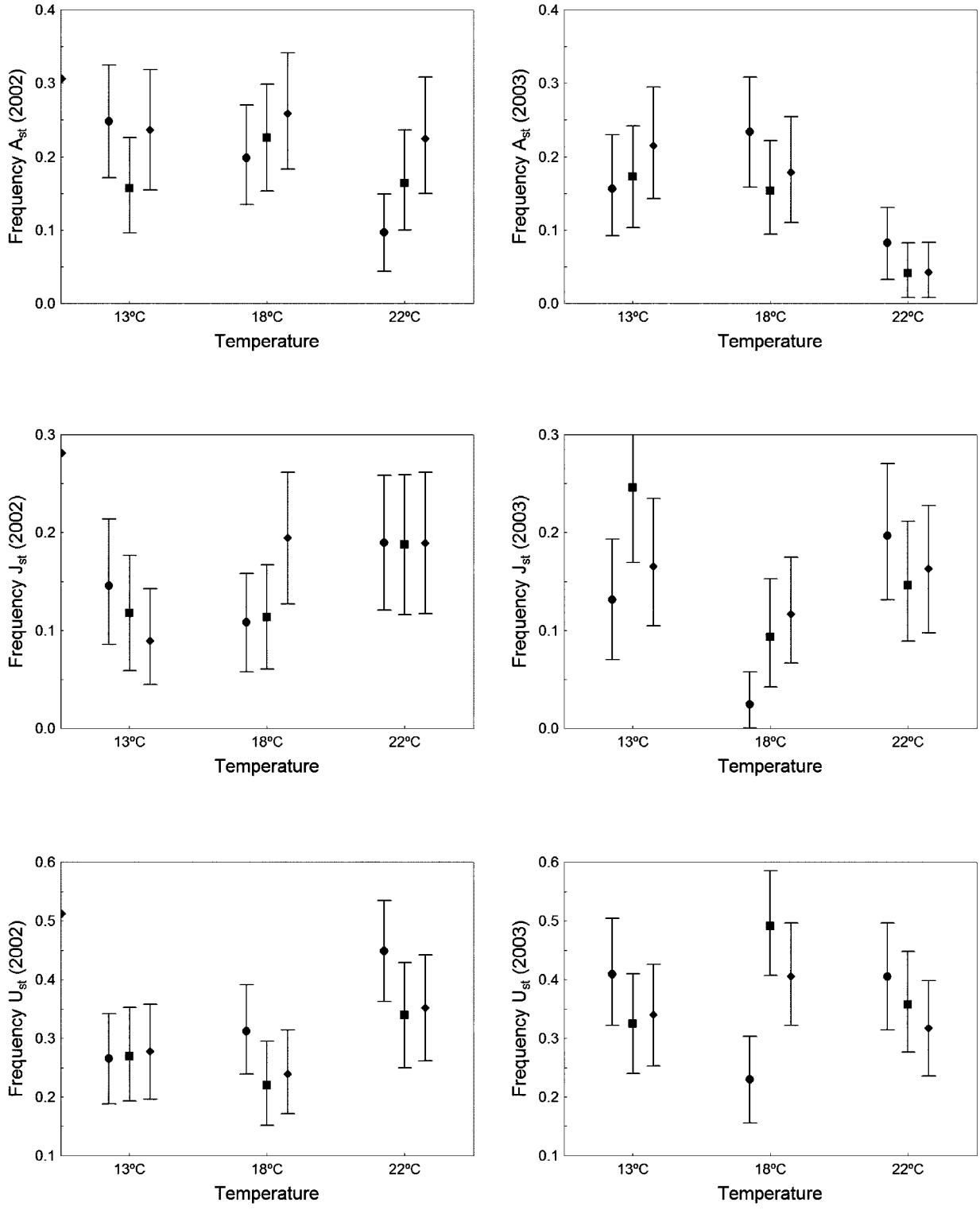


Figure 2: Average frequencies (with 95% confidence intervals) of standard gene arrangements after 1 (May 2002: *left panels*) and 2 years (April 2003: *right panels*) of thermal selection (*filled circles* = R1, *filled squares* = R2, *filled diamonds* = R3). Paired panels (left-right) are plotted at the same scale for easier contrasts. The small arrows in the left panels point to the corresponding initial frequency in the founding population derived from Puerto Montt (Chile). Confidence intervals were calculated by using the bootstrap percentile method after 10,000 bootstrap samples (Efron and Tibshirani 1993).

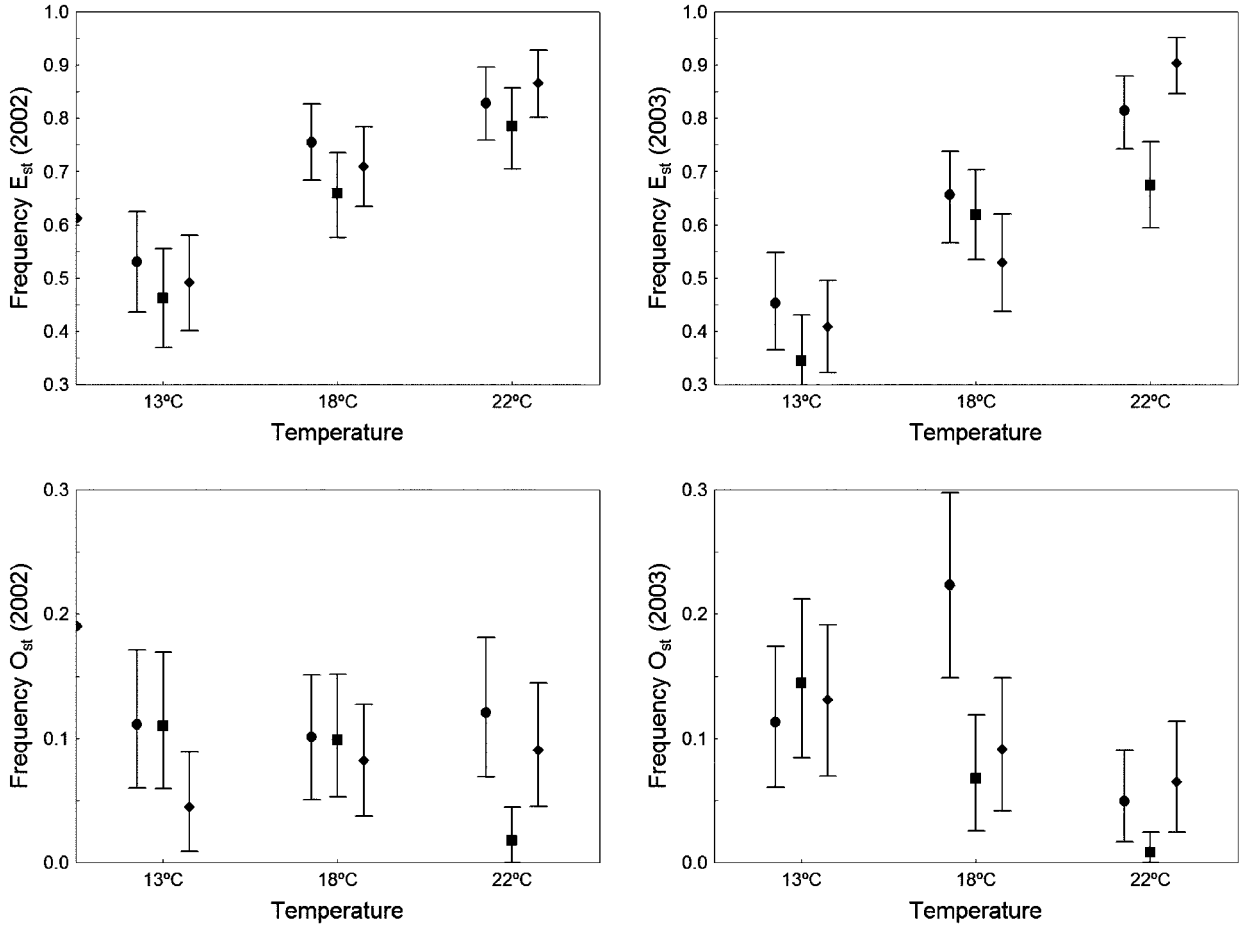
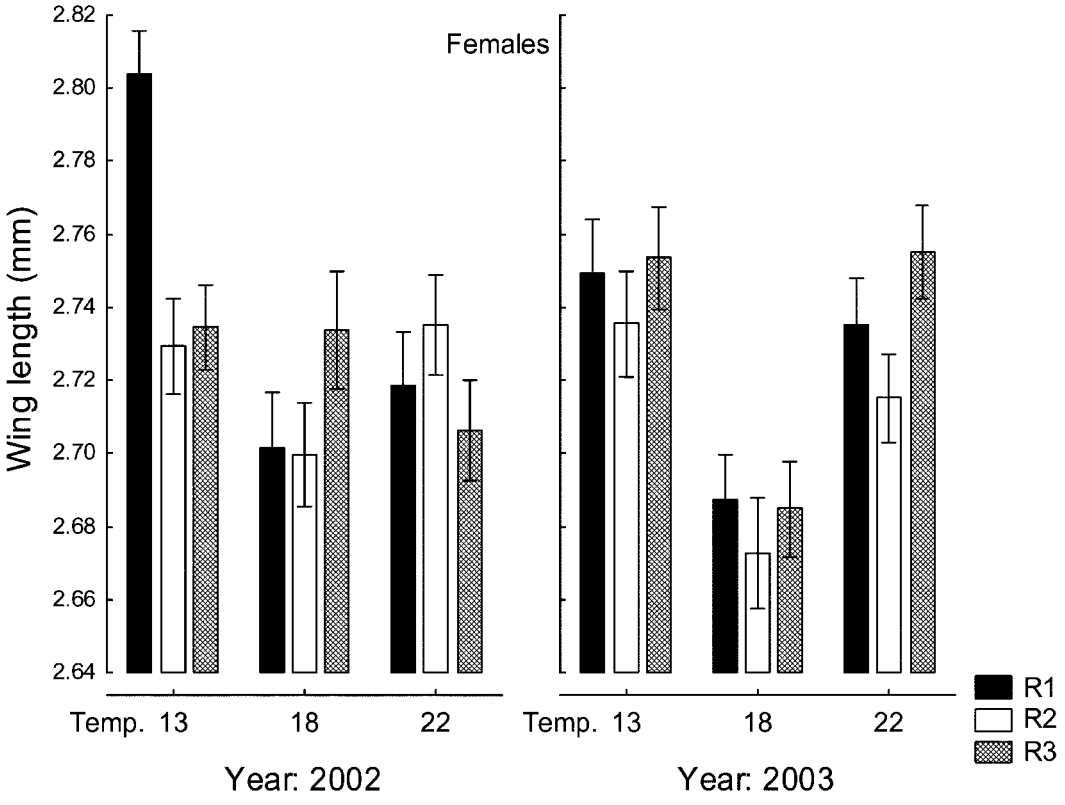


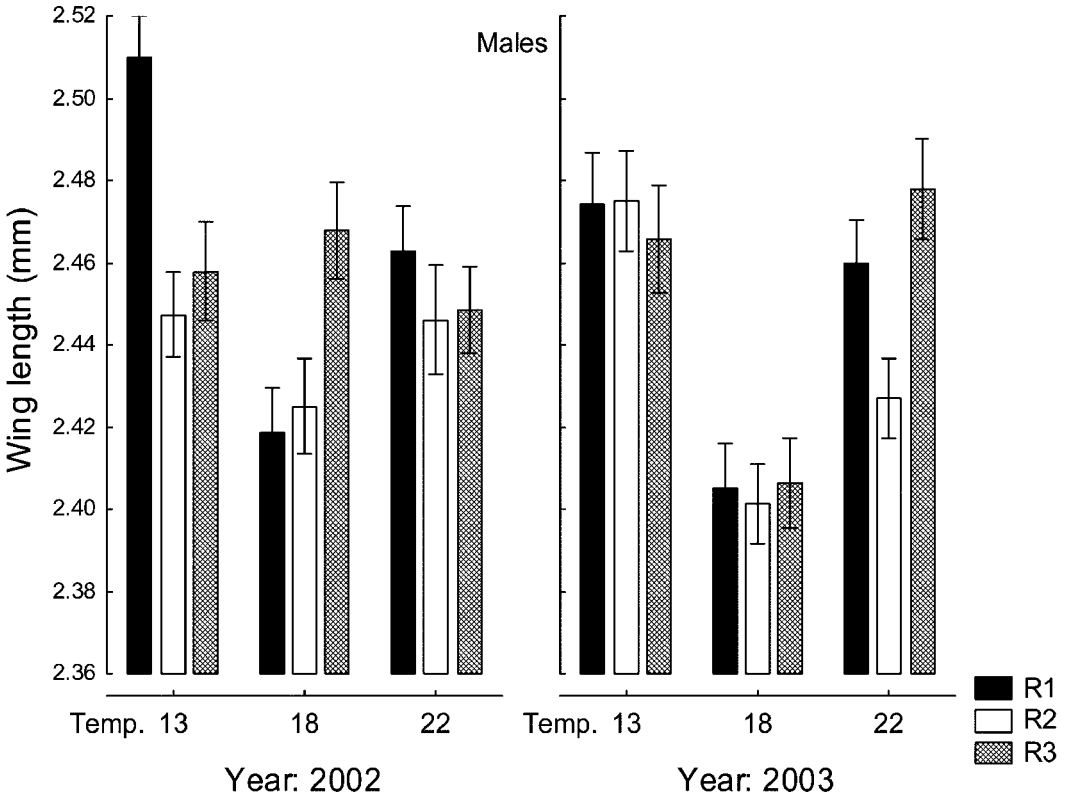
Figure 2 (*Continued*)

licated populations within each thermal regime has decreased at 18°C and remained quite unchanged at 13°C and 22°C. In fact, two-way ANOVAs with temperature and year as the main fixed effects, and replicate populations nested within temperature, indicated a highly significant temperature × year interaction effect (females: $F = 15.75$, df $= 2, 1{,}783$, $P < .001$; males: $F = 17.66$, df $= 2, 2{,}096$, $P < .001$). However, orthogonal contrasts showed that adult flies from the populations at 13°C (females May 2002 [2.7560 mm] vs. April 2003 [2.7462], $P = .094$; males May 2002 [2.4721] vs. April 2003 [2.4717], $P = .942$) and 22°C (females May 2002 [2.7200 mm] vs. April 2003 [2.7352], $P = .008$; males May 2002 [2.4526 mm] vs. April 2003 [2.4548], $P = .589$) had indeed about the same average WL in both samples (most remarkable in the case of males). If anything has happened between the two samples at the two extreme temperatures, then female's size seems to have decreased at the lowest temperature and increased at the highest one.

Cell Size and Cell Number Index. The random sample of 50 wings per sex and population used to estimate the cellular components of body size differentiation showed the same pattern for WL as that plotted in figure 3 for the whole sample (females: $F = 22.44$, df $= 2, 6$, $P = .002$; males: $F = 13.43$, df $= 2, 6$, $P = .006$). Two-level nested ANOVAs of cell area did not detect statistical significant differences among temperatures (females: $F = 3.00$, df $= 2, 6$, $P = .125$; males: $F = 1.36$, df $= 2, 6$, $P = .326$; see fig. 4*A*), which at first glance would suggest that the aforementioned reduction in wing size at the optimum thermal selection regime was mainly brought about by a decrease in the number of cells in the wing blade. However, the average number of cells (cell number index) was also roughly the same across thermal treatments (females: $F = 0.94$, df $= 2, 6$, $P = .441$; males: $F = 2.29$, df $= 2, 6$, $P = .182$; see fig. 4*B*).

A principal component (PC) analysis for cell size/cell


Females
Wing length (mm)
2.82
2.80
2.78
2.76
2.74
2.72
2.70
2.68
2.66
2.64
Temp. 13
18
22
Year: 2002
Year: 2003
R1
R2
R3


Males
Wing length (mm)
2.52
2.50
2.48
2.46
2.44
2.42
2.40
2.38
2.36
Temp. 13
18
22
Year: 2002
Year: 2003
R1
R2
R3

number clearly defined an inverse relationship between both variables. Female loadings are

$$\text{PC1} = \begin{pmatrix} -2.77 \times 10^{-3} \\ 1 \end{pmatrix},$$

$$\text{PC2} = \begin{pmatrix} 1 \\ 2.77 \times 10^{-3} \end{pmatrix}.$$

Male loadings are

$$\text{PC1} = \begin{pmatrix} -2.45 \times 10^{-3} \\ 1 \end{pmatrix},$$

$$\text{PC2} = \begin{pmatrix} 1 \\ 2.45 \times 10^{-3} \end{pmatrix}.$$

Two-level nested ANOVAs on PC2 (or the computed z scores) detected statistically significant differences among temperatures (females: $F = 10.27$, df $= 2, 6$, $P = .012$; males: $F = 10.97$, df $= 2, 6$, $P = .010$). Overall, it seems that the ~2% reduction in wing size at the optimum temperature of 18°C was mediated by both cell area and cell number. However, the response in cell area was more important in females while the opposite appears to be true in males. In fact, a two-way ANOVA for PC2 with temperature and sex as the main fixed effects, and replicate populations nested within temperature, rendered a statistically significant temperature × sex interaction ($F = 3.02$, df $= 2, 888$, $P = .049$).

Relationship between Chromosomal Polymorphism and Wing Size and Shape Index

Sample sizes from the thermal stocks amount to more than 2,000 males for which we have simultaneous information on chromosome arrangements and wing dimensions. Three-way ANOVAs for WL, with temperature, year, and gene arrangement as the main fixed effects, and replicate populations nested within temperature are shown in table 1. The relevant effects here are those for gene arrangement and all its interaction terms. In no case were these interaction terms statistically significant, thus suggesting quite consistent results across thermal regimes and samples for the effects of chromosome arrangements on wing length.

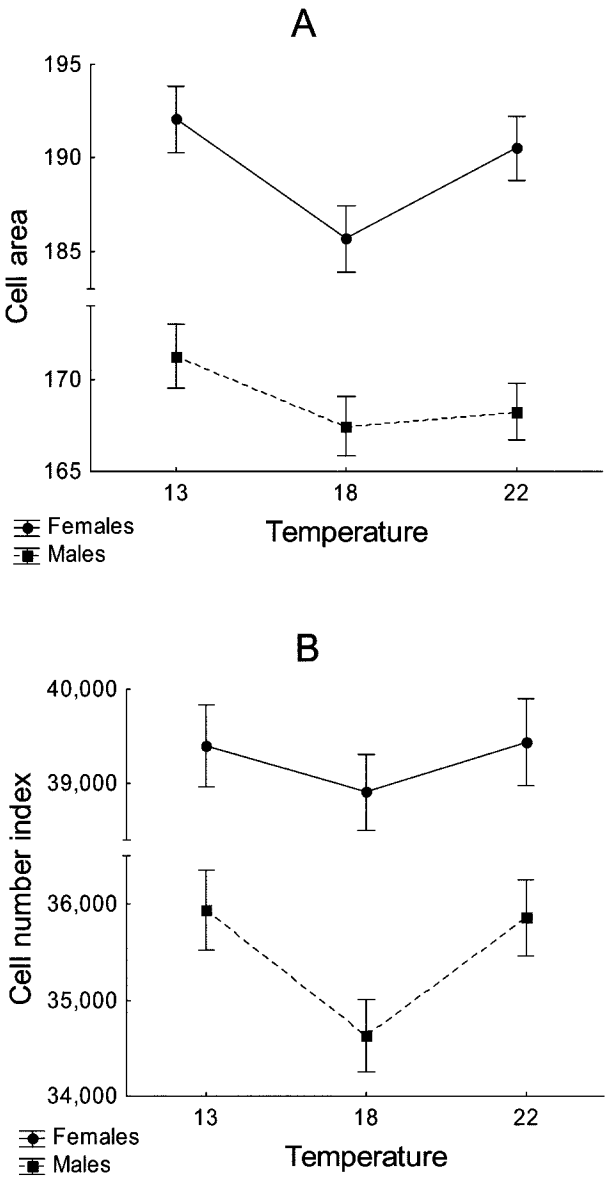


Figure 4: Means and 95% confidence intervals for (*A*) cell area (values in μm^2) and (*B*) cell number index from the thermal selection stocks when reared at 18°C under uncrowded conditions. To enhance visibility, all replicate populations within temperature have been pooled.

Figure 3: Means and 95% confidence intervals for wing length of *Drosophila subobscura* females (*top*) and males (*bottom*) from the thermal selection stocks when reared at 18°C under uncrowded conditions. Data from 2002 had been previously published (Santos et al. 2004) and are plotted here for comparisons. Female sample sizes in 2003 were the following: 13°C, R1 = 98, R2 = 100, R3 = 100; 18°C, R1 = 100, R2 = 100, R3 = 100; 22°C, R1 = 98, R2 = 99, R3 = 100. Male sample sizes were the following: 13°C, R1 = 115, R2 = 116, R3 = 111; 18°C, R1 = 113, R2 = 115, R3 = 117; 22°C, R1 = 114, R2 = 120, R3 = 119.

Table 1: Three-way ANOVAs for the wing length of *Drosophila subobscura* males

	Chromosome A		Chromosome J		Chromosome U		Chromosome E		Chromosome O[a]	
Source	df	MS	df	MS	df	MS	df	MS	df	MS
Temperature (T)	2	5.024*	2	4.076*	2	7.015*	2	3.602*	2	.816*
Replicate ⊂ (T)	6	.975***	6	1.007***	6	1.017***	6	.979***	6	1.012***
Year (Y)	1	.613**	1	.391*	1	1.154***	1	.558**	1	.065 NS
Arrangement (A)	1	.709**	1	.002 NS	2	.084 NS	3	.773***	5	.244**
T × Y	2	.584***	2	.551***	2	1.196***	2	.417**	2	.252*
T × A	2	.152 NS	2	.119 NS	4	.092 NS	6	.056 NS	10	.033 NS
Y × A	1	.003 NS	1	.014 NS	2	.134 NS	3	.036 NS	5	.044 NS
T × Y × A	2	.000 NS	2	.017 NS	4	.121 NS	6	.065 NS	10	.097 NS
Error	2,009	.067	2,073	.067	2,065	.067	2,061	.066	2,048	.066

Note: ANOVAs performed to test for biometrical effects of the polymorphic gene arrangements on the major five acrocentric chromosomes. WL as $\log_e$(L1 + L2) in pixels. MS × 10^{-2}; ⊂ = nested in; NS = not significant at $P > .05$.

[a] The recombination-derived O_7 gene arrangement was excluded from the analysis.

* $P < .05$.

** $P < .01$.

*** $P < .001$.

Polymorphic inversions on chromosomes A, E, and O seem to have a statistically significant effect on body size with the following patterns (after Scheffé post hoc tests where appropriate): $A_{st} < A_2$, $E_{1+2+9+3} < E_{st} = E_{1+2+9+12}$, $O_{3+4+8} < O_{3+4+2}$. The only case where a standard gene arrangement was associated with a larger body size when compared to a nonstandard one was for chromosome E, but arrangement $E_{1+2+9+3}$ shows a quite flat relationship with latitude in New World populations (see Balanyà et al. 2003).

In the May 2002 sample, we had detected a significant and consistent (across thermal selection regimes) reduction of the wing shape index L1/WL with increasing standard dose, that is, with the number of standard gene arrangements carried out by a male, which ranges from 0 to 5 (Santos et al. 2004). A three-way ANOVA for L1/WL (as $\log_e$[L1/WL]), with temperature, year, and standard dose (0, 1, 2, and ≥3) as the main fixed effects, and replicate populations nested within temperature revealed that none of the interaction terms involving the standard dose were statistically significant (results not shown). Therefore, the previously reported trend of a relative shortening of the basal length of longitudinal vein IV with an increase of the standard dose in *D. subobscura* flies from the New World is highly reliable.

Discussion

Replicate thermal selection stocks of *Drosophila subobscura* flies derived from the estimated epicenter of the original New World invasion and maintained on a discrete generation, controlled larval crowding regime showed, first, a lower chromosomal diversity at the warmest and suboptimal temperature; second, a quick and consistent shift in gene arrangement frequencies in response to thermal selection regime; third, an evolutionary decrease in wing size—mediated by both cell area and cell number—at the optimum temperature of the species; fourth, no positive relationship between wing size and the standard gene arrangements on the five major acrocentric chromosomes; and fifth, a shortening of the basal length of longitudinal vein IV relative to its total length (i.e., wing shape index) with increasing standard dose. In the following, we discuss these findings in relation to patterns found in natural populations and results from earlier laboratory thermal selection experiments with various *Drosophila* species.

Patterns of Chromosomal Diversity

At first glance, the negative relationship we found between chromosomal diversity and the evolutionary effect of temperature is against the geographic distribution of 1 − IFR in the Old World. This index decreases in a southeastern (Asia Minor, Balkans)–north-northwestern (Scandinavia, Scotland) direction from 0.30 to 0.10, but with some spots of relatively high diversity (0.20) in Northeastern Spain (see Krimbas 1992, p. 194). This Palearctic geographic pattern (traditionally interpreted in terms of ecological "centrality" or "marginality"; see Krimbas and Loukas 1980), however, might bear little relationship with temperature adaptation and could be simply explained as a postglacial population expansion from a Balkan refugium. Actually, from a number of European case studies (e.g., the common meadow grasshopper of Europe, *Chorthippus parallelus*), it appears that the Balkans—as well as Italy, Spain, and Turkey, which also contained refugia for several species—were a source for all species in the east and for many species in the west (Hewitt 2000, 2001).

Particularly, the Southern Balkan peninsula harbors some *D. subobscura* gene arrangements specific for that refugium (e.g., E_8, U_{1+2+6}) in high frequencies (Menozzi and Krimbas 1992). Thus, the standing geographic patterns of distribution in the Old World could only mirror historical processes and do not easily allow inferring current adaptation.

Inversion Clines and Laboratory Cage Results

The results of inversion frequency shifts after the New World invasion by *D. subobscura* were clear: clines fully developed within the first 5 years or so after colonization, and evolution was predictable (Balanyà et al. 2003). However, what remains largely unknown is whether such patterns were driven primarily by abiotic or biotic processes.

Because our thermal stocks were initiated from an ancestral population sampled at the estimated Chilean epicenter of the original New World invasion, these results should be more appropriately contrasted against the New World latitudinal clines summarized in Balanyà et al. (2003). The most obvious feature was a general lack of correspondence between the outcomes from laboratory thermal selection and New World colonizations: the only case where both laboratory and natural trends were coincident is for arrangement O_{3+4+2} (and perhaps O_{st}). For chromosome U, no trends were detected in the thermal stocks, while clear latitudinal clines are evident in New World populations with U_{st} increasing and U_{1+2+8} decreasing with increasing latitude. On the other hand, for chromosome E, the trends observed in the thermal stocks were just opposite of those observed in nature, where arrangement E_{st} sharply increased in frequency with latitude soon after colonization. What seems to be quite coherent, however, is that by and large the chromosomal inversion polymorphism quickly responded to laboratory temperature: several gene arrangements with no latitudinal trends also showed a flat relationship with experimental temperature.

Certainly, laboratory experiments are not the best way to reconstruct natural clines. Our experimental controlled conditions obviously do not mirror the nutritional status or the daily and seasonal ranges in temperature experienced by flies in the field and might have been unsuccessful at identifying the real impact of temperature on polymorphic inversions (see Mitrovski and Hoffmann 2001; Hoffman et al. 2003). With these caveats in mind, however, we could tentatively conclude that the natural patterns do not seem to be generally caused by a direct effect of temperature. Previous laboratory cage experiments (summarized in Krimbas 1993) had also reached similar conclusions, but results were usually inconsistent because lines were started with different and often genetically homogeneous base populations. Inversion-related selective dispersal (see Gosteli 1991), karyotype by environment interactions of selection in wild populations, and/or selective agents correlated with latitude may work together to generate clinal patterns in New World colonizing populations.

Clinal Variation of Body Size and Inversion Polymorphism

In contrast to what had been observed for chromosome arrangements, wing size latitudinal clines were not evident in either North or South America about 1 decade after the introduction of *D. subobscura* (Budnik et al. 1991; Pegueroles et al. 1995), but it took ~20 years for them to evolve on both continents (Huey et al. 2000; Gilchrist et al. 2001, 2004; Calboli et al. 2003*a*). This plain fact already suggests that body size in New World populations was likely unrelated to those gene arrangements implicated in the latitudinal clines, which is against some claims based on European samples that point to a positive association between wing size and standard gene arrangement dose (Prevosti 1967; Orengo and Prevosti 2002; but see Krimbas 1967).

Correlations between body size and polymorphic inversions have certainly been observed for different *Drosophila* species. Thus, in *Drosophila buzzatii*, gene arrangements on chromosome 2 have a worldwide consistent biometrical effect on body size (Betrán et al. 1998), and in *Drosophila melanogaster*, some cosmopolitan inversions on the major autosomal arms have also been reported to affect size-related traits (e.g., Stalker 1980; van Delden and Kamping 1991). Indeed, the recently published quantitative trait loci (QTL) mapping results by Gockel et al. (2002) and Calboli et al. (2003*b*) show that 77% of the underlying latitudinal clinal variance of *D. melanogaster* body size in Australia and South America (see also Gockel et al. 2001) is explained by the third chromosome; additionally, the highest QTL peaks, explaining almost completely the variance due to that chromosome, maps on the inversion *In(3R)Payne*, which itself shows clinal variation (see Knibb et al. 1981; Knibb 1982; Weeks et al. 2002). Similar interactions with inversion *In(2L)t* in quantitative-trait clines from Australia and the west coast of South America are also quite likely (see James et al. 1995; van't Land et al. 2000). These results forcefully stress the difficulty of disentangling the effects of *Drosophila* polymorphic inversions on the clinal variation in quantitative traits (e.g., body size). Our present data, however, clearly show that New World latitudinal clines for polymorphic inversions and body size (but not wing shape; see Santos et al. 2004 and above) in *D. subobscura* are uncoupled. Obviously, this finding does not rule out the possibility that the long-standing body size cline in the Old World (Prevosti 1955; Misra and Reeve 1964; Pfriem 1983; Pegueroles et al. 1995) could certainly be correlated with the clines in chromo-

somal arrangement frequencies, and, therefore, the lack of correlation in samples from the New World might be a by-product of a founder effect that occurred during the colonization. In any case, it is worth mentioning that the autosomes that seem to have an effect on body size in *D. subobscura* (E and O) are homologous to those that have been consistently implicated in the control of body size in *D. melanogaster* latitudinal clines (2R and 3R, respectively; Gockel et al. 2002; Calboli et al. 2003*b*) and in *D. buzzatii* (chromosome 2 is homologous to chromosome O).

Laboratory Thermal Evolution of Body Size

Warm-adapted populations (22°C) were not smaller than their cold-adapted (13°C) counterparts and had about the same cell size (fig. 4*A*). It could be claimed that this result might be due to the relatively short duration of the selection experiment because thermal evolution of body size in *Drosophila* had been previously shown in populations kept at various temperatures for long periods of time (>4 years; Anderson 1973; Cavicchi et al. 1989; Partridge et al. 1994*a*). However, the comparative body size reduction of ~2% observed at the optimum temperature of 18°C (fig. 3; an incipient but nonsignificant decrease was already observed in the first sample) shows that an evolutionary response of wing size has already occurred. This reduction was mediated by both cell area and cell number. A quite general result of laboratory thermal selection experiments is that differences in wing area are entirely a consequence of cell size divergence (Robertson 1959; Cavicchi et al. 1985; Partridge et al. 1994*a*). However, our results seem to confirm Partridge et al.'s (1999) findings where a correlated response to decreasing size was mediated by both cell area and cell number.

As argued earlier, a common oversight in thermal selection experiments was the lack of control of larval culture density across temperatures. A crowded environment can cause high mortality at the egg, larval, and pupal stages. As the food is consumed, harmful waste products also accumulate (Borash et al. 1998), suggesting that growth rate will decline over time. Selection is expected to favor more competitive larva, which would likely cause a faster deterioration of the culture, as suggested by the results of Joshi and Mueller (1996) and Santos et al. (1997), showing that populations adapted to larval crowding consumed more food but used it less efficiently. Therefore, the additional results that cold-adapted populations have a greater efficiency of conversion of food to biomass and reduced competitive ability (Partridge et al. 1994*b*; Neat et al. 1995) could be explained to some extent by the common observation that populations of *D. melanogaster* stabilize at lower densities when maintained at lower temperatures. Although there is no clear theoretical prediction concerning the evolution of body size (Mueller 1988), the outcomes might be dependent on the larval crowding regime. Thus, populations adapted to very high levels of larval crowding show about the same size at eclosion when assayed at low densities than their control counterparts (Santos et al. 1997). However, selection at moderate larval densities may result in an evolutionary decline of body size (Roper et al. 1996). Wolf (2003) has recently shown that a considerable portion of the genetic variation for body size in *Drosophila* may result from interactions among individuals and has also suggested that genetic variation for competitive ability appears as genetic variation for body size. Furthermore, maintenance at high larval densities led to the evolution of greater population growth rates at high density (Mueller and Ayala 1981), and there is suggestive evidence that adaptations to larval versus adult crowding trade off in *D. melanogaster* (Joshi et al. 1998; Borash and Ho 2001). Overall, the overwhelming evidence showing that density-dependent natural selection affects important life-history characteristics (reviewed in Mueller 1997) certainly sets important hurdles on our understanding of thermal adaptation per se in *Drosophila.*

As far as we are aware, there are only three previous laboratory thermal selection experiments carried out in *D. melanogaster* that have controlled for larval densities. Cavicchi et al. (1985, 1989) set up three populations reared at three different temperatures and maintained them for more than 4 years by placing 10 random pairs in each of four bottles containing abundant food. Unfortunately, the number of breeding adults per population was quite low, and the lines were not replicated, so genetic drift cannot be ruled out as the cause of the population divergence (notice from fig. 3 the large variation in body size among our replicates). More recently, Bochdanovits and de Jong (2003*a*) have analyzed the interaction between temperature and food quality regimes by setting four experimental evolution lines of *D. melanogaster* at four different combinations. They found that thermal evolution of body size interacts with the food quality regime; however, lines were kept with a relatively low number of breeding adults and were not replicated, so it would be highly desirable to repeat those experiments. Last, W. J. Kennington and J. Gockel (unpublished results) did not find any size differentiation after 55 generations of thermal selection under standardized larval crowding. Even though the interplay between temperature and larval density has never been critically addressed, we think that two clear conclusions emerge when all the information is taken together: first, larval crowding seems to play an important role in the establishment of body size differences in thermal selection experiments and may also play a part in the establishment of body size clines (see also Partridge and French 1996);

second, it is not necessarily true that adaptation at low temperature per se invariably results in the evolution of a larger body size in *Drosophila*. New experiments manipulating both temperature and larval density are highly needed.

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Appendix from M. Santos et al., "Temperature-Related Genetic Changes in Laboratory Populations of *Drosophila subobscura*: Evidence against Simple Climatic-Based Explanations for Latitudinal Clines"

(Am. Nat., vol. 165, no. 2, p. 258)

Tables of Chromosomal Polymorphism and Wing Figure

Table A1
Chromosomal polymorphism of *Drosophila subobscura*

	Chromosome A			Chromosome J		
Sample	A_{st}	A_2	*N*	J_{st}	J_1	*N*
Puerto Montt (1999)	68 (50.75)	66 (49.25)	134	41 (30.37)	94 (69.63)	135
Foundation (2001)	37 (30.58)	84 (69.42)	121	34 (28.10)	87 (71.90)	121
13°C R1 (2002)	29 (24.79)	88 (75.21)	117	17 (14.53)	100 (85.47)	117
13°C R2 (2002)	18 (24.66)	97 (36.06)	115	14 (11.76)	105 (88.24)	119
13°C R3 (2002)	26 (35.62)	84 (31.23)	110	10 (8.93)	102 (91.07)	112
18°C R1 (2002)	25 (19.84)	101 (80.16)	126	15 (10.79)	124 (89.21)	139
18°C R2 (2002)	28 (22.58)	96 (77.42)	124	15 (11.36)	117 (88.64)	132
18°C R3 (2002)	31 (25.83)	89 (74.17)	120	26 (19.40)	108 (80.60)	134
22°C R1 (2002)	11 (9.65)	103 (90.35)	114	22 (18.97)	94 (81.03)	116
22°C R2 (2002)	18 (16.36)	92 (83.64)	110	21 (18.75)	91 (81.25)	112
22°C R3 (2002)	24 (22.43)	83 (77.57)	107	21 (18.92)	90 (81.08)	111
13°C R1 (2003)	17 (15.60)	92 (84.40)	109	15 (13.16)	99 (86.84)	114
13°C R2 (2003)	20 (17.24)	96 (82.76)	116	29 (24.58)	89 (75.42)	118
13°C R3 (2003)	24 (21.43)	88 (78.57)	112	19 (16.52)	96 (83.48)	115
18°C R1 (2003)	28 (23.33)	92 (76.67)	120	3 (2.46)	119 (97.54)	122
18°C R2 (2003)	18 (15.38)	99 (84.62)	117	11 (9.32)	107 (90.68)	118
18°C R3 (2003)	21 (17.80)	97 (82.20)	118	14 (11.67)	106 (88.33)	120
22°C R1 (2003)	10 (8.20)	112 (91.80)	122	24 (19.67)	98 (80.33)	122
22°C R2 (2003)	5 (4.13)	116 (95.87)	121	18 (14.63)	105 (85.37)	123
22°C R3 (2003)	5 (4.17)	115 (95.83)	120	20 (16.26)	103 (83.74)	123

Sample	Chromosome U				Chromosome E[a]				
	U_{st}	U_{1+2}	U_{1+2+8}	*N*	E_{st}	$E_{1+2+9+3}$	E_{1+2+9}	$E_{1+2+9+12}$	*N*
Puerto Montt (1999)	57 (42.22)	47 (34.81)	31 (22.96)	135	80 (59.26)	20 (14.81)	8 (5.93)	22 (16.30)	135
Foundation (2001)	62 (51.24)	34 (28.10)	25 (20.66)	121	74 (61.16)	28 (23.14)	9 (7.44)	10 (8.26)	121
13°C R1 (2002)	31 (26.50)	63 (53.85)	23 (19.66)	117	62 (52.99)	25 (21.37)	4 (3.42)	26 (22.22)	117
13°C R2 (2002)	32 (26.89)	57 (47.90)	30 (25.21)	119	55 (46.22)	20 (16.81)	8 (6.72)	36 (30.25)	119
13°C R3 (2002)	31 (27.68)	54 (48.21)	27 (24.11)	112	55 (49.11)	18 (16.07)	3 (2.68)	36 (32.14)	112
18°C R1 (2002)	43 (31.16)	73 (52.90)	22 (15.94)	138	105 (75.54)	14 (10.07)	5 (3.60)	15 (10.79)	139
18°C R2 (2002)	29 (21.97)	78 (59.09)	25 (18.94)	132	87 (65.91)	10 (7.58)	13 (9.85)	22 (16.67)	132
18°C R3 (2002)	32 (23.88)	69 (51.49)	33 (24.63)	134	95 (70.90)	24 (17.91)	3 (2.24)	12 (8.96)	134
22°C R1 (2002)	52 (44.83)	41 (35.34)	23 (19.83)	116	96 (82.76)	5 (4.31)	13 (11.21)	2 (1.72)	116
22°C R2 (2002)	38 (33.93)	49 (43.75)	25 (22.32)	112	88 (78.57)	13 (11.61)	5 (4.46)	6 (5.36)	112
22°C R3 (2002)	39 (35.14)	53 (47.75)	19 (17.12)	111	96 (86.49)	7 (6.31)	5 (4.50)	3 (2.70)	111
13°C R1 (2003)	47 (40.87)	38 (33.04)	30 (26.09)	115	52 (45.22)	33 (28.70)	10 (8.70)	20 (17.39)	115
13°C R2 (2003)	38 (32.48)	44 (37.61)	35 (29.91)	117	40 (34.48)	29 (25.00)	11 (9.48)	36 (31.03)	116
13°C R3 (2003)	39 (33.91)	36 (31.30)	40 (34.78)	115	47 (40.87)	29 (25.22)	3 (2.61)	36 (31.30)	115
18°C R1 (2003)	28 (22.95)	49 (40.16)	45 (36.89)	122	80 (65.57)	23 (18.85)	11 (9.02)	8 (6.56)	122
18°C R2 (2003)	58 (49.15)	40 (33.90)	20 (16.95)	118	73 (61.86)	15 (12.71)	10 (8.47)	20 (16.95)	118
18°C R3 (2003)	49 (40.50)	29 (23.97)	43 (35.54)	121	64 (52.89)	29 (23.97)	13 (10.74)	15 (12.40)	121
22°C R1 (2003)	49 (40.50)	43 (35.54)	29 (23.97)	121	101 (81.45)	15 (12.10)	6 (4.84)	2 (1.61)	124
22°C R2 (2003)	44 (35.77)	48 (39.02)	31 (25.20)	123	83 (67.48)	18 (14.63)	13 (10.57)	9 (7.32)	123
22°C R3 (2003)	39 (31.71)	52 (42.28)	32 (26.02)	123	111 (90.24)	7 (5.69)	3 (2.44)	2 (1.63)	123

Sample	Chromosome O							
	O_{st}	O_{3+4+2}	O_{3+4}	O_5	O_{3+4+7}	O_{3+4+8}	O_7	*N*
Puerto Montt (1999)	39 (28.89)	34 (25.19)	11 (8.15)	10 (7.41)	17 (12.59)	23 (17.04)	1 (.74)	135
Foundation (2001)	23 (19.01)	51 (42.15)	6 (4.96)	6 (4.96)	17 (14.05)	18 (14.88)	0 (.00)	121
13°C R1 (2002)	13 (11.11)	68 (58.12)	8 (6.84)	0 (.00)	10 (8.55)	18 (15.38)	0 (.00)	117
13°C R2 (2002)	13 (10.92)	70 (58.82)	4 (3.36)	1 (.84)	17 (14.29)	13 (10.92)	1 (.84)	119
13°C R3 (2002)	5 (4.46)	70 (62.50)	9 (8.04)	1 (.89)	13 (11.61)	14 (12.50)	0 (.00)	112
18°C R1 (2002)	14 (10.07)	86 (61.87)	10 (7.19)	0 (.00)	15 (10.79)	14 (10.07)	0 (.00)	139
18°C R2 (2002)	13 (9.85)	80 (60.61)	6 (4.55)	0 (.00)	15 (11.36)	18 (13.64)	0 (.00)	132
18°C R3 (2002)	11 (8.21)	67 (50.00)	10 (7.46)	1 (.75)	24 (17.91)	21 (15.67)	0 (.00)	134
22°C R1 (2002)	14 (12.07)	62 (53.45)	21 (18.10)	0 (.00)	5 (4.31)	14 (12.07)	0 (.00)	116
22°C R2 (2002)	2 (1.79)	51 (45.54)	29 (25.89)	2 (1.79)	14 (12.50)	14 (12.50)	0 (.00)	112
22°C R3 (2002)	10 (9.01)	61 (54.95)	21 (18.92)	0 (.00)	12 (10.81)	7 (6.31)	0 (.00)	111
13°C R1 (2003)	13 (11.30)	67 (58.26)	5 (4.35)	0 (.00)	12 (10.43)	18 (15.65)	0 (.00)	115
13°C R2 (2003)	17 (14.41)	73 (61.86)	9 (7.63)	1 (.85)	9 (7.63)	9 (7.63)	0 (.00)	118
13°C R3 (2003)	15 (13.04)	69 (60.00)	6 (5.22)	0 (.00)	15 (13.04)	10 (8.70)	0 (.00)	115
18°C R1 (2003)	27 (22.13)	64 (52.46)	8 (6.56)	1 (.82)	14 (11.48)	7 (5.74)	1 (.82)	122
18°C R2 (2003)	8 (6.78)	70 (59.32)	6 (5.08)	1 (.85)	17 (14.41)	16 (13.56)	0 (.00)	118
18°C R3 (2003)	11 (9.09)	73 (60.33)	7 (5.79)	0 (.00)	13 (10.74)	17 (14.05)	0 (.00)	121
22°C R1 (2003)	6 (4.92)	80 (65.57)	17 (13.93)	1 (.82)	8 (6.56)	10 (8.20)	0 (0)	122
22°C R2 (2003)	1 (.81)	85 (69.11)	13 (10.57)	0 (.00)	4 (3.25)	20 (16.26)	0 (.00)	123
22°C R3 (2003)	8 (6.50)	80 (65.04)	17 (13.82)	0 (.00)	9 (7.32)	9 (7.32)	0 (.00)	123

Note: Data are from populations at Puerto Montt (November 1999), the founding population (May 2001), and the thermal selection stocks after 1 (May 2002: nine generations at 13°C, 12 at 18°C, and 15 at 22°C) and 2 years (April 2003: 16 generations at 13°C, 22 at 18°C, and 29 at 22°C) of thermal selection (proportions for each gene arrangement are given in parentheses).

[a] Gene arrangement E_{1+2} was also present at Puerto Montt ($n = 5$; 3.70%) but was likely lost in the founding population and is not included in the table.

Table A2
Two-level nested ANOVAs for the indicator variable x_{ij}

	Chromosome A				Chromosome J				Chromosome U			
	A_{st} (2002)		A_{st} (2003)		J_{st} (2002)		J_{st} (2003)		U_{st} (2002)		U_{st} (2003)	
Source	df	MS	df	MS	df	MS	df	MS	df	MS	df	MS
Temperature	2	.43	2	2.01**	2	.46	2	1.14	2	1.62*	2	.03
Regression	1	.41	1	2.61**	1	.83*	1	.06	1	1.86*	1	.00
Deviation	1	.44	1	1.42*	1	.09	1	2.23*	1	1.39	1	.06
Replicate	6	.28	6	.12	6	.13	6	.25	6	.24	6	.87**
Error	1,034	.16	1,046	.12	1,083	.13	1,066	.12	1,082	.21	1,066	.23
Slope (β)	…		−.0136 ± .0029		.0077 ± .0030		…		.0112 ± .0039		…	
β-pooled[a]	−.0098 ± .0022				NS				NS			

	Chromosome E				Chromosome O[b]			
	E_{st} (2002)		E_{st} (2003)		O_{st} (2002)		O_{st} (2003)	
Source	df	MS	df	MS	df	MS	df	MS
Temperature	2	9.77***	2	13.99**	2	.03	2	.93
Regression	1	19.32***	1	27.88**	1	.02	1	1.32
Deviation	1	.23	1	.10	1	.03	1	.54
Replicate	6	.21	6	.82***	6	.17	6	.33**
Error	1,083	.20	1,068	.21	1,082	.08	1,067	.09
Slope (β)	.0372 ± .0038		.0438 ± .0038		…		…	
β-pooled[a]	.0404 ± .0027				NS			

Note: $x_{ij} = 1$ if chromosome arrangement j in male i was standard and $x_{ij} = 0$ otherwise. The slopes of arrangement frequencies on temperature (±SE after grouping the three replicated populations within temperatures) are given whenever the linear effects (regression) were statistically significant. Statistical significance was computed after 10,000 random permutations (see text for details). NS = not significant. $P > .05$.

[a] Homogeneity of slopes ($P > .05$) was tested from the temperature × year interaction effect.

[b] The recombination-derived O_7 gene arrangement was excluded from the analyses.

* $P < .05$.

** $P < .01$.

*** $P < .001$.

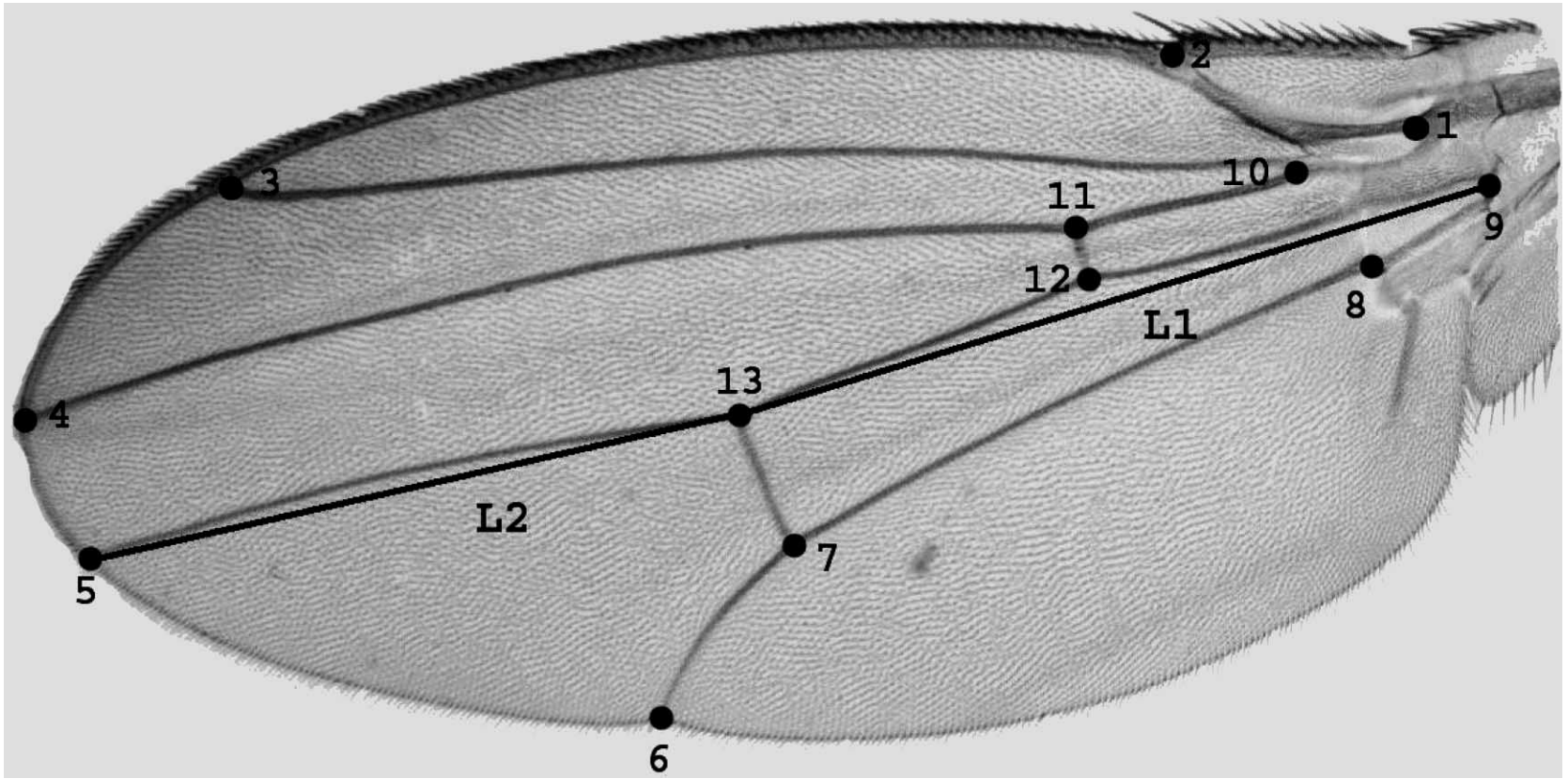

Figure A1: Image of *Drosophila subobscura* left wing indicating the 13 landmarks used in this work. The lengths of the proximal (*L1*) and distal (*L2*) segments of longitudinal vein IV were calculated as the euclidean distance between landmarks 9 and 13 and landmarks 13 and 5, respectively.

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Research article

Genetics and geometry of canalization and developmental stability in *Drosophila subobscura*

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Abstract

Background: Many properties of organisms show great robustness against genetic and environmental perturbations. The terms canalization and developmental stability were originally proposed to describe the ability of an organism to resist perturbations and to produce a predictable target phenotype regardless of random developmental noise. However, the extent to which canalization and developmental stability are controlled by the same set of genes and share underlying regulatory mechanisms is largely unresolved.

Results: We have analyzed the effects of clinal genetic variation (inversion polymorphism) on wing asymmetry by applying the methods of geometric morphometrics in the context of quantitative genetics using isochromosomal lines of *Drosophila subobscura*. For the analysis of overall size, developmental stability was positively correlated with levels of heterozygosity and development at the optimal temperature. For analyses of shape, the overall comparisons by matrix correlations indicate that inter- and intraindividual variation levels were poorly correlated, a result also supported when comparing the vectors describing patterns of variation of landmark position. The lack of similarity was basically due to the discrepancy between the genetic and environmental components of the interindividual variation. Finally, the analyses have also underscored the presence of genetic variation for directional asymmetry.

Conclusions: The results strongly support the hypothesis that environmental canalization and developmental stability share underlying regulatory mechanisms, but environmental and genetic canalization are not functionally the same. A likely explanation for this lack of association is that natural wing shape variation in *Drosophila* populations is loosely related to individual fitness.

Background

Phenotypic robustness refers to the invariance of the specified target phenotype given the genetic makeup and environmental conditions. Whereas the presence of naturally occurring phenotypic variation is at the core of evolutionary biology, developmental geneticists have traditionally considered it as a nuisance. Instead, they have relied on the study of single or multiple mutant combinations to reveal the generation of phenotypic patterns (e.g. [1]). A resurgence of interest in the issue of phenotypic robustness has emerged in recent years, partly due to experimental results showing that many knock-out mutations have

little effect on phenotype ([2]; although Papp's et al. [3] metabolic network analysis found that the majority of genes that looked dispensable turn out to be such only under laboratory conditions), and that developmental systems show a high degree of stability with respect to perturbations [4,5].

Three major processes are involved in the control of phenotypic variability (the potential or propensity to vary, in the terminology of Wagner and Altenberg [6]): canalization, developmental stability (DS), and plasticity [7]. As first defined by Waddington [8] the term canalization could be understood as a morphogenetic constrain [9], where development appears to be buffered so that slight abnormalities of genotype or slight perturbations in the environment do not lead to the production of abnormal phenotypes. However, evolutionary geneticists define canalization as the tendency of traits to evolve a reduction in variability [4,10]. DS can be defined as the ability of organisms to buffer against the random noise that arises spontaneously as a consequence of stochastic variation in the cellular processes that are involved in the development of morphological structures [11]. Therefore, canalization and DS are subcategories of developmental buffering: the first can be appraised by estimating interindividual variance whereas the most commonly used estimate of DS in bilaterally symmetrical organisms is fluctuating asymmetry (FA); i.e. the intraindividual variation due to random differences between left and right sides. The question of whether or not canalization and DS are different buffering mechanisms has been a constant source of debate. Two recent reviews implicitly [4] or explicitly [10] assume that DS is a special case of canalization, a viewpoint also embraced by several authors (e.g. [12-14]). Thus, by using geometric morphometrics Klingenberg and McIntyre [13] found that the vectors describing inter- and intraindividual variation of landmark position for fly vein traits were highly concordant. On the other hand, Debat et al. [15] came to the opposite conclusion applying the same methods to cranial landmarks in the house mouse – although Klingenberg's et al. [16] work with mouse mandibles found patterns of intra- and interindividual variation that were only partly consistent –. At first glance, the different results may suggest that the mechanisms that affect canalization and DS are related in some developmental contexts but not in others. The problem is, however, that according to the causes of phenotypic variation a distinction between genetic and environmental canalization is necessary [17,18]. Selection for environmental canalization may produce genetic canalization as a by-product [4,10], but this may not always be the case.

The better way to address these contentious issues is to rely on quantitative genetic analyses devised to partition phenotypic variation into genetic and environmental components [19]. Environmental variation can be further partitioned into general (σ^2_E) and special (micro) environmental effects (σ^2_e): the first refer to influential factors (e.g. temperature) that are shared by groups of individuals, whereas the latter are residual deviations from the phenotype that would be specified on the basis of genotype and general environmental effects. Such deviations are unique to individuals and are largely unpredictable. The variance associated with special environmental effects can be estimated when experiments are performed on completely inbred lines (i.e., there is no genetic variance). In bilaterally symmetrical organisms it is also feasible to estimate the two sources that contribute to those special environmental effects: among-individual (σ^2_{ea}) and within-individual variance (σ^2_{ew}). If the only real cause of asymmetry is variation due to stochasticity in development, then FA can be taken as an estimated of σ^2_{ew}. Therefore, FA is only one source of the phenotypic variation within environments (excluding environmentally induced asymmetry), contrarily to the arguments in Nijhout and Davidovitz [20]. The other source is σ^2_{ea}.

The third process involved in the control of phenotypic variability is plasticity, which can be defined as the ability of an individual to express one phenotype under one set of environmental circumstances and another phenotype under another set. The expressed phenotypes can be discontinuous thus eliciting discrete morphs (i.e., polyphenism), or there can be a continuous range of potential phenotypes (i.e., reaction norm). The reaction norm is thus a property of the genome: genetic canalization and phenotypic plasticity are not mutually exclusive and can combine to form canalized reaction norms [7,17]. Plasticity is thus an alternative to genetic change allowing populations to adapt to changing environmental conditions. To summarize, phenotypic plasticity increases the variance among groups of individuals that produce different phenotypes in different environments, canalization decreases the within-group interindividual variance around the target phenotype by reducing the sensitivity to genetic and environmental conditions, and DS buffers against random perturbations in development (i.e., decreases FA). Because the left and right body sides share the same genome (barring unusual somatic mutation or somatic recombination) and in most organisms also very nearly the same environment, FA provides an intrinsic control for genetic and environmental effects and the important question is to what extent these two sources of variation share underlying regulatory mechanisms.

Within the framework of recently developed geometrically based methods for the statistical analysis of size and shape variation (collectively referred to as geometric morphometrics [21,22]), the wing vein network of *Drosophila* is regarded as an excellent model system to investigate those problems [23,24]. Wing development in *Drosophila* is well understood [25], and the vein pattern is highly conserved across species (e.g. [26]). When flies are reared at low temperatures it is well known that the final wing size increases because of an increase in adult cell size [27]. This plastic response is parallel to what has been commonly observed in laboratory experiments on thermal evolution, where adaptation to lower temperature resulted in increased wing size (a proxy for body size) entirely as a consequence of cell size divergence [28]. However, there is circumstantial evidence suggesting that developmental and evolutionary temperature-related cell size divergence have contrasting effects on wing shape. Thus, Birdsall et al. [29] concluded that wing shape in *Drosophila melanogaster* is quite resistant to developmental temperature. Conversely, in *D. subobscura* there are changes in wing proportions along a latitudinal size cline mediated by cell area [30,31]. These populations exhibit, in addition, prominent latitudinal clines for chromosomal inversion polymorphisms, and there is compelling evidence showing that the inversion clines underlie the latitudinal changes in wing proportions [32,33].

Here we report on the effects of clinal genetic variation (inversion polymorphism) on wing form (size and shape) and bilateral asymmetry using isochromosomal lines of *D. subobscura*. We consider the consequences of inbreeding and temperature on the two components of developmental homeostasis (canalization and DS), and the relationship between them. The remainder of the paper is planned as follows. First, we provide a short account of the inversion polymorphism in *D. subobscura* and the experimental settings. Then, based on the well balanced data set rendered by the experimental design we used the standard least-squares (ANOVA) method to decompose sources of variation for wing size and shape into causal components at the core of further analyses. Furthermore, because the underlying assumption to use FA as a measure of DS is that left – right-side variation has not heritable basis, the genetic and environmental components of bilateral asymmetry were partitioned. As a result, our approach is unusual in studies of DS in providing estimates of the two components of special environmental effects (co-) variance under different genetic backgrounds and general environmental settings. We also present some evidence for the presence of genetic variation in directional asymmetry (DA) but not in FA. Next, we test whether or not the vectors describing variation of landmark position for fly vein traits are concordant, and finally we discuss the main findings in relation with the evolution of buffering mechanisms and the putative adaptive value of natural wing shape variation in *D subobscura*.

Experimental settings

D. subobscura is a particularly inversion-rich species, with up to 38 natural chromosomal arrangements already reported for the largest chromosome O (homologous to arm 3R in *D. melanogaster* [34]) for which a balancer stock is available. In colonizing populations of the New World only six gene arrangements are segregating for that chromosome: O_{st}, O_{3+4}, O_{3+4+2}, O_{3+4+7}, O_{3+4+8} and O_5 (arrangement O_7 is also present at very low frequency but it is probably the result of a recombination event in the O_{st}/O_{3+4+7} heterokaryotype [35]). In native Palearctic populations arrangements O_{3+4+2} and O_{3+4+8} are restricted to the Mediterranean region (the likely area from which the original American colonists derived [36]) and are not involved in latitudinal clines [35]. On the other hand, arrangement O_{st} shows a world-wide positive correlation with latitude, while arrangements O_{3+4} and O_{3+4+7} show a contrasting pattern [35]. Therefore, six independent isochromosomal lines for each of these three chromosome arrangements (i.e., O_j^1, ..., O_j^6; j = st, 3+4, 3+4+7) were used in the present experiments.

The experimental flies were obtained from 54 crosses, which will be referred to as inbred (isogenic; i.e., $O_j^1 \times O_j^1$, $O_j^2 \times O_j^2$,..., $O_j^6 \times O_j^6$) with 18 crosses in total, or outbred (including both structural homo- and heterokaryotypes) with 36 (18 + 18) crosses in total. The six lines with a given gene arrangement were crossed to produce the three different outbred homokaryotypes (i.e., $O_j^1 \times O_j^2$, $O_j^2 \times O_j^3$,..., $O_j^6 \times O_j^1$). The three kinds of heterokaryotypic flies were similarly obtained but using lines with different gene arrangements (i.e., $O_j^1 \times O_k^1$, $O_j^2 \times O_k^2$,..., $O_j^6 \times O_k^6$; $j \neq k$). Since all isochromosomal lines were homogeneous for the same genetic background (except for the male sex chromosome), maternal effects were not considered to be critically important. Anyhow, experimental flies were randomly derived form reciprocal crosses for all outbred combinations. Two developmental temperatures were used in the experiment: optimal (18°C) and warm (23°C).

Results and discussion

Variation and asymmetry in size

a) Basic statistics

Signed left-right ($\bar{L} - \bar{R}$) differences of centroid size did not significantly departure from normality in any case (D_{max} ranging from 0.032 for inbred females at 18°C to 0.073 for inbred males at 23°C; $P > 0.05$). In addition,

Table 1: Asymmetry of overall wing size for females raised at 18°C *Drosophila subobscura* flies raised from inbred (isogenic) and outbred crosses reared at 18°C. Centroid size (CS, estimated in a normalized form [22]) is the dependent variable (values in pixels²: 1 mm = 144 pixels). The ANOVAs assess measurement error, directional asymmetry (Sides effect), fluctuating asymmetry (Individuals × Sides interaction effect), and genetic components of the trait (σ_g^2) and DA of the trait (σ_g^2 (DA)). σ_w^2 (CS) and σ_w^2 (DA_{CS}) provide here unbiased estimates of the among-fly (i.e. σ_{ea}^2) and within-fly (σ_{ew}^2 or FA) special environmental effects. (⊂ means 'nested in'.)

		Inbred			Outbred		
Source of variation	Variance component	d.f.	Mean Square	Estimated variance	d.f.	Mean Square	Estimated variance
Individuals (I)		107	39.747***	9.6175	215	45.773***	11.2830
Karyotypes (K)	σ_k^2 (CS)	2	114.593n.s.	0.1389	5	214.204n.s.	0.7014
Cross ⊂ K	σ_l^2 (CS)	15	94.589***	2.7352	30	113.199***	3.4726
Among flies	σ_w^2 (CS)	90	28.944***	6.9166	180	29.857***	7.3040
Sides (S)		1	15.982***		1	18.549***	
I × S	$\sigma_{I\times S}^2$ (CS)	107	1.278***	0.5467	215	0.641***	0.2225
Karyotypes (K)	σ_k^2 (DA_{CS})	2	0.067n.s.	-0.0520	5	0.457n.s.	-0.0123
Cross ⊂ K	σ_l^2 (DA_{CS})	15	1.938¶	0.1239	30	0.899¶	0.0492
Within flies	σ_w^2 (DA_{CS})	90	1.194***	0.5051	180	0.603***	0.2036
Measurement error	σ_m^2 (CS)	216	0.184	0.1841	432	0.196	0.1962

Average CS for left (*L*) and right (*R*) wings: inbred females $\overline{L}$ = 0.9918 mm, $\overline{R}$ = 0.9891 ; outbred females $\overline{L}$ = 1.0022, $\overline{R}$ = 1.0002.
n.s. $P > 0.10$; ¶ $0.10 > P > 0.05$; *** $P < 0.001$.

none of the regressions of centroid size FA on average wing size was statistically significant (ranging from β = -0.045 (95% C.I.: -0.091, 0.001) for inbred females at 18°C to β = 0.030 (-0.005, 0.064) for inbred females at 23°C), thus suggesting independence between size and size FA.

b) Causal components of variation

For each sex two-way mixed ANOVAs were separately performed for inbred and outbred crosses at each experimental temperature (Tables 1, 2, 3, 4). Size variation (CS: centroid size) among individuals comprised the largest part (> 90%) of the variation. The fraction of the total phenotypic variance in wing size associated to genetic differences and/or lines (i.e., $\frac{\sigma_k^2(CS)+\sigma_l^2(CS)}{\sigma_k^2(CS)+\sigma_l^2(CS)+\sigma_w^2(CS)}$) ranged from 0.235 (inbred females at 23°C). (Bear in mind that there is nothing in the ANOVA method of estimation that will prevent a negative variance estimate [37].)

No significant size differences were generally detected among karyotypes for average CS, although O_{3+4} flies were always the biggest within inbred lines (Fig. 1). On the other hand, in outbred crosses heterokaryotypes were bigger than homokaryotypes (females: 18°C $F_{(1,195)}$ = 9.78, P = 0.002; 23°C $F_{(1,195)}$ = 9.19, P = 0.003; males: 18°C $F_{(1,195)}$ = 1.84, P = 0.176; 23°C $F_{(1,195)}$ = 4.23, P = 0.041), but interactions of dominance effects were observed in all samples with discernible heterosis in O_{st}/O_{3+4} lines when compared to their homokaryotypic counterparts.

In concert with some independent preliminary results using a set of O_{st} isochromosomal lines [38] a quite remarkable finding here was that left wings were consistently bigger than the right ones, thus causing a generally highly significant DA (i.e., "sides" effect in Tables 1, 2, 3, 4) of overall wing size even though DA was fairly subtle (see bottom statistics in Tables 1, 2, 3, 4). Each *Drosophila* wing vein has dorsal and ventral components that come together after the apposition of the dorsal and ventral surfaces, but each vein protrudes only in one wing surface ("corrugation") [25]. When wings were mounted no attempt was made to standardize the surface position: in females 394 (60.8%) left and 387 (59.7%) right wings were mounted on the slides with the dorsal side up (χ^2 =

Table 2: Asymmetry of overall wing size for males raised at 18°C Same as in Table 1.

			Inbred			Outbred	
Source of variation	Variance component	d.f.	Mean Square	Estimated variance	d.f.	Mean Square	Estimated variance
Individuals (I)		107	43.303***	10.4842	215	38.600***	9.4200
Karyotypes (K)	σ_k^2 (CS)	2	232.359¶	1.1331	5	115.718$^{n.s.}$	0.1908
Cross ⊂ K	σ_l^2 (CS)	15	69.186*	1.4332	30	88.246***	2.5026
Among flies	σ_w^2 (CS)	90	34.788***	8.3554	180	28.184***	6.8159
Sides (S)		1	1.140$^{n.s.}$		1	22.492***	
I × S	$\sigma_{I\times S}^2$ (CS)	107	1.366***	0.5045	215	0.920***	0.3297
Karyotypes (K)	σ_k^2 (DA_{CS})	2	0.385$^{n.s.}$	-0.0176	5	1.156$^{n.s.}$	0.0035
Cross ⊂ K	σ_l^2 (DA_{CS})	15	1.017$^{n.s.}$	-0.0715	30	1.031$^{n.s.}$	0.0226
Within flies	σ_w^2 (DA_{CS})	90	1.446***	0.5444	180	0.895***	0.3172
Measurement error	σ_m^2 (CS)	216	0.357	0.3574	432	0.261	0.2609

Average CS for left (*L*) and right (*R*) wings: inbred males $\overline{L}$ = 0.8942, $\overline{R}$ = 0.8935; outbred males $\overline{L}$ = 0.9003, $\overline{R}$ = 0.8980.
$^{n.s.}$ $P > 0.10$; ¶ $0.10 > P > 0.05$; * $P < 0.05$; *** $P < 0.001$.

Table 3: Asymmetry of overall wing size for females raised at 23°C Same as in Table 1 for *Drosophila subobscura* flies reared at 23°C

			Inbred			Outbred	
Source of variation	Variance component	d.f.	Mean Square	Estimated variance	d.f.	Mean Square	Estimated variance
Individuals (I)		107	64.857***	15.8796	215	49.100***	11.9347
Karyotypes (K)	σ_k^2 (CS)	2	27.808$^{n.s.}$	-1.8893	5	457.293***	2.6178
Cross ⊂ K	σ_l^2 (CS)	15	299.873***	11.3901	30	80.332***	1.9907
Among flies	σ_w^2 (CS)	90	26.511***	6.2931	180	32.556***	7.7987
Sides (S)		1	33.413***		1	16.825***	
I × S	$\sigma_{I\times S}^2$ (CS)	107	1.339***	0.5446	215	1.361***	0.5916
Karyotypes (K)	σ_k^2 (DA_{CS})	2	0.681$^{n.s.}$	-0.0125	5	4.333*	0.0781
Cross ⊂ K	σ_l^2 (DA_{CS})	15	1.132$^{n.s.}$	-0.0427	30	1.520$^{n.s.}$	0.0447
Within flies	σ_w^2 (DA_{CS})	90	1.388***	0.5692	180	1.252***	0.5371
Measurement error	σ_m^2 (CS)	216	0.250	0.2496	432	0.178	0.1778

Average CS for left (*L*) and right (*R*) wings: inbred females $\overline{L}$ = 0.8999 mm, $\overline{R}$ = 0.8960; outbred females $\overline{L}$ = 0.9203, $\overline{R}$ = 0.9184.
$^{n.s.}$ $P > 0.10$; * $P < 0.05$; *** $P < 0.001$.

Table 4: Asymmetry of overall wing size for males raised at 23°C Same as in Table 1 for *Drosophila subobscura* flies reared at 23°C

			Inbred			Outbred	
Source of variation	Variance component	d.f	Mean Square	Estimated variance	d.f.	Mean Square	Estimated variance
Individuals (I)		107	44.690***	10.9045	215	28.772***	6.8138
Karyotypes (K)	σ_k^2 (CS)	2	41.926$^{n.s.}$	-0.6021	5	112.284$^{n.s.}$	0.3480
Cross ⊂ K	σ_l^2 (CS)	15	128.628***	4.0778	30	62.165***	1.7199
Among flies	σ_w^2 (CS)	90	30.762***	7.4224	180	20.887***	4.8425
Sides (S)		1	36.691***		1	13.586**	
I × S	$\sigma_{I\times S}^2$ (CS)	107	1.072***	0.3553	215	1.517***	0.6465
Karyotypes (K)	σ_k^2 (CS)	2	2.596$^{n.s.}$	0.0366	5	0.862$^{n.s.}$	-0.0110
Cross ⊂ K	σ_l^2 (DA_{CS})	15	1.277$^{n.s.}$	0.0454	30	1.259$^{n.s.}$	-0.0532
Within flies	σ_w^2 (DA_{CS})	90	1.004***	0.3213	180	1.578***	0.6771
Measurement error	σ_m^2 (CS)	216	0.361	0.3615	432	0.224	0.2240

Average CS for left (*L*) and right (*R*) wings: inbred males $\overline{L}$ = 0.8112, $\overline{R}$ = 0.8072 ; outbred males $\overline{L}$ = 0.8277, $\overline{R}$ = 0.8260.
$^{n.s.}$ $P > 0.10$; ** $P < 0.01$; *** $P < 0.001$.

0.16, 1 df, $P = 0.691$); in males the corresponding figures were 383 (59.1%) and 401 (61.9%), respectively (χ^2 = 1.05, 1 df, $P = 0.306$). Potential biasing effects when measuring wings; namely, dorsal or ventral Bitmap images or possible differences between left and right wings when Bitmap images are captured from the top or bottom of the microscope slide, were checked from a subset of 75 females and 75 males. An additional set of two images for each wing were taken in the same session from the top and bottom of the slide and digitized once. The centroid size differences between the averages of both measurements was apparently random with respect to digitizing procedure and always lower than 0.07%, whereas left wings were 0.26% bigger than the right ones in females and 0.34% in males. We are, therefore, quite confident that the fairly subtle DA for wing CS is not an experimental artifact but a real phenomenon.

In addition to DA, there was subtle but significant FA in all crosses (i.e., "individuals × sides" interaction effect in Tables 1, 2, 3, 4) together with a small amount of genetic variation for DA in some of them. This last finding could hardly be attributable to a type I error because similar results had been previously obtained [[38]; see below]. Conversely, two-level nested ANOVAs to test for genetic components of overall size FA (using index FA1 in Palmer [39]) failed to show any statistically significant effects whatsoever (variance components ranging from -0.0047 to 0.0071 for karyotypes, and from -0.0343 to 0.0406 for crosses within karyotypes; values in pixels^2).

c) Consanguinity and temperature effects

Inbreeding and environmental effects were simultaneously analyzed by contrasting isogenic *vs*. outbred homokaryotypic flies reared at both experimental temperatures (Fig. 1). Flies were obviously bigger when raised at the lowest temperature, and three-way factorial ANOVAs performed separately for each sex using CS (as $\log_e$ (pixels), but results were qualitatively identical without a log-transformation) as the dependent variable, with karyotype, temperature and inbreeding as fixed effects, and crosses nested within karyotypes, clearly indicated inbreeding depression together with temperature by inbreeding interaction (i.e., inbreeding was most noticeable at the sub-optimal temperature of 23°C), but no karyotype by temperature interaction was detected. These results confirm that wing size is not a purely additive trait in *D. subobscura*, in agreement with the previous observation that heterokaryotypes were bigger than homokaryotypes in outbred crosses (see also [40]).

Both inbreeding and (sub-optimal) temperature effects were also apparent in females when overall size FA (index FA1) was used as the dependent variable in three-way fac-

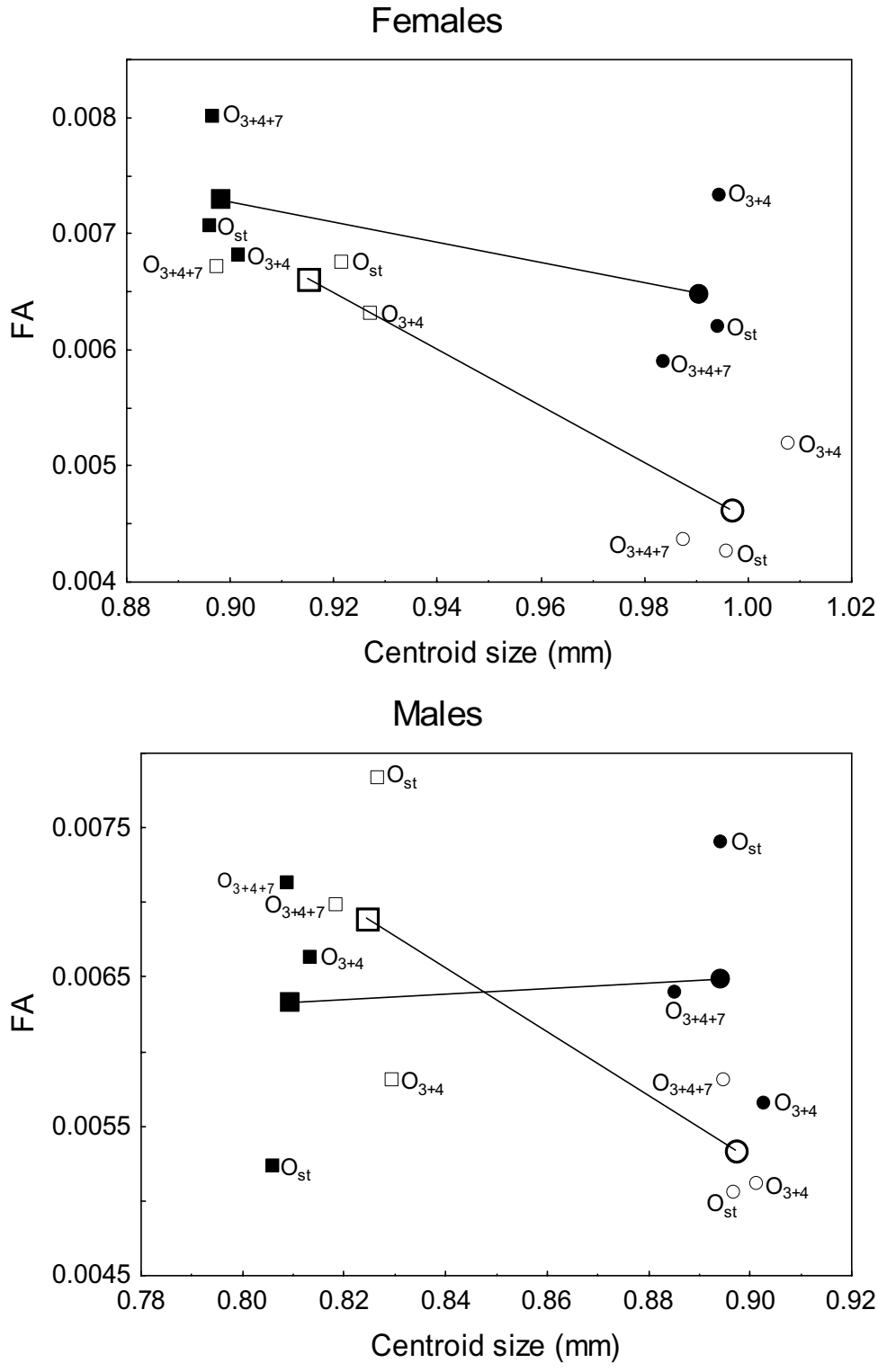

Figure 1
Inbreeding and temperature effects on size Homokaryotipic averages for centroid size and centroid size FA (index FA1 in [39]) in inbred (black symbols) and outbred (open symbols) crosses. Small symbols give the average values for each of the three different homokaryotypes to appreciate the dispersion from the corresponding grand average (large symbols connected by lines). Squares give the values at 23°C and circles at 18°C.

torial ANOVAs, with no differences among karyotypes. On the other hand, no statistically significant effects were detected for males, basically because inbred crosses performed approximately equal at both temperatures (Fig. 1). However, overall asymmetry augmented in inbred crosses because DA largely increased (mainly in males) at the highest temperature ("temperature × inbreeding" interaction: $F_{(1,856)} = 9.46$, $P = 0.002$).

It is worth mentioning here that in outbred crosses overall size FA was about the same for homokaryotypes and heterokaryotypes: the only significant effect was again an increase in FA at the sub-optimal temperature (more than two-fold; c.f. σ^2_w (DA_{CS}) values in Tables 1, 2, 3, 4). Finally, inbreeding appears to have affected among-fly variation only in males as suggested by the consistently lower σ^2_w (CS) estimates in outbred crosses within rearing temperature.

In conclusion, overall size DS was positively correlated with levels of heterozygosity (i.e., inbred vs. outbred homokaryotypes) and development at the optimal temperature of 18°C. However, no positive association was found between DS and chromosomal heterozygosity in outbred crosses.

Table 5: Asymmetry of overall wing shape for females raised at 18°C Flies raised from inbred (isogenic) and outbred crosses of *Drosophila subobscura* reared at 18°C. For the inbred crosses 15 PC scores were retained for analyses (proportion of total shape variance accounted is given in parenthesis). For the outbred crosses 22 PC scores were retained. (⊂ means 'nested in'.)

	Inbred (98.6%)				Outbred			
Source of variation	Wilks' lambda	df 1	df 2	*P*	Wilks' lambda	df 1	df 2	*P*
Individuals (I)	1.13×10^{-11}	1605	1464	<0.001	5.14×10^{-15}	4730	4442	<0.001
Karyotypes (K)	0.002	30	2	0.505	7.44×10^{-5}	110	48	<0.001
Cross ⊂ K	1.51×10^{-4}	225	843	<0.001	3.68×10^{-4}	660	2932	<0.001
Among flies	2.23×10^{-8}	1350	1457	<0.001	7.55×10^{-12}	3960	4430	<0.001
Sides (S)	0.597	15	93	<0.001	0.563	22	194	<0.001
I × S	1.58×10^{-9}	1605	3083	<0.001	6.46×10^{-11}	4730	9192	<0.001
Karyotypes (K)	0.003	30	2	0.546	0.002	110	48	0.301
Cross ⊂ K	0.074	225	843	0.362	0.018	660	2932	0.023
Within flies	9.69×10^{-9}	1350	3070	<0.001	7.91×10^{-10}	3960	9169	<0.001

Table 6: Asymmetry of overall wing shape for males raised at 18°C Same as in Table 5.

	Inbred (98.5%)				Outbred			
Source of variation	Wilks' lambda	df 1	df 2	*P*	Wilks' lambda	df 1	df 2	*P*
Individuals (I)	7.18×10^{-12}	1605	1464	<0.001	3.51×10^{-14}	4730	4442	<0.001
Karyotypes (K)	0.004	30	2	0.633	2.49×10^{-4}	110	48	0.006
Cross ⊂ K	1.61×10^{-4}	225	843	<0.001	2.98×10^{-4}	660	2932	<0.001
Among flies	1.47×10^{-8}	1350	1457	<0.001	3.62×10^{-11}	3960	4430	<0.001
Sides (S)	0.658	15	93	<0.001	0.569	22	194	<0.001
I × S	5.58×10^{-8}	1605	3083	<0.001	1.28×10^{-10}	4730	9192	<0.001
Karyotypes (K)	0.004	30	2	0.605	0.003	110	48	0.449
Cross ⊂ K	0.068	225	843	0.236	0.019	660	2932	0.036
Within flies	3.05×10^{-7}	1350	3070	<0.001	1.73×10^{-9}	3960	9169	<0.001

Table 7: Asymmetry of overall wing shape for females raised at 23°C Same as in Table 5 for *Drosophila subobscura* flies reared at 23°C.

	Inbred (98.3%)				Outbred			
Source of variation	Wilks' lambda	df 1	df 2	*P*	Wilks' lambda	df 1	df 2	*P*
Individuals (I)	1.07×10^{-12}	1605	1464	<0.001	1.08×10^{-13}	4730	4442	<0.001
Karyotypes (K)	2.18×10^{-4}	30	2	0.200	0.001	110	48	0.146
Cross ⊂ K	3.31×10^{-4}	225	843	<0.001	1.81×10^{-4}	660	2932	<0.001
Among flies	2.32×10^{-9}	1350	1457	<0.001	1.54×10^{-10}	3960	4430	<0.001
Sides (S)	0.450	15	93	<0.001	0.585	22	194	<0.001
I × S	2.57×10^{-9}	1605	3083	<0.001	3.21×10^{-13}	4730	9192	<0.001
Karyotypes (K)	0.007	30	2	0.725	0.006	110	48	0.842
Cross ⊂ K	0.055	225	843	0.062	0.034	660	2932	0.889
Within flies	1.95×10^{-8}	1350	3070	<0.001	3.54×10^{-12}	3960	9169	<0.001

Table 8: Asymmetry of overall wing shape for males raised at 23°C Same as in Table 5 for *Drosophila subobscura* flies reared at 23°C.

	Inbred (98.3%)				Outbred			
Source of variation	Wilks' lambda	df 1	df 2	*P*	Wilks' lambda	df 1	df 2	*P*
Individuals (I)	5.39×10^{-12}	1605	1464	<0.001	6.41×10^{-14}	4730	4442	<0.001
Karyotypes (K)	8.81×10^{-4}	30	2	0.364	1.18×10^{-4}	110	48	<0.001
Cross ⊂ K	2.75×10^{-4}	225	843	<0.001	1.96×10^{-4}	660	2932	<0.001
Among flies	8.92×10^{-9}	1350	1457	<0.001	7.94×10^{-11}	3960	4430	<0.001
Sides (S)	0.642	15	93	<0.001	0.540	22	194	<0.001
I × S	8.58×10^{-9}	1605	3083	<0.001	9.84×10^{-12}	4730	9192	<0.001
Karyotypes (K)	5.13×10^{-5}	30	2	0.102	6.40×10^{-4}	110	48	0.052
Cross ⊂ K	0.060	225	843	0.111	0.024	660	2932	0.250
Within flies	5.79×10^{-8}	1350	3070	<0.001	1.26×10^{-10}	3960	9169	<0.001

Variation and asymmetry in shape

a) Sources of variation

Two-way MANOVA analyses to quantify inter- and intra-individual variation in wing shape are shown in Tables 5, 6, 7, 8. For the present study of 13 landmarks, with 2 coordinates each, the shape dimension is 22. Sums of squares and cross-products (SSCP) matrices are therefore not full-ranked, and we retained 22 PC (principal components [41]) scores for outbred crosses and only 15 PC scores – which accounted for more than 98% of the total shape variance – for inbred crosses to be capable of testing for genetic components. The degrees of freedom in Tables 5, 6, 7, 8 (columns "df 1") are simply the corresponding degrees of freedom in the ANOVAs for centroid size (Tables 1, 2, 3, 4) times the number of PC scores retained in each sample. Likewise, the overall covariation in wing shape ("individuals" effect) was decomposed into causal components (karyotypes, crosses in karyotypes, and among flies); and the overall covariation in wing shape FA ("individuals × sides" interaction effect) was decomposed into causal components attributable to wing shape DA (karyotypes, crosses in karyotypes, and within flies).

Similarly to what had been found for CS, differences between left and right wings were also highly significant ("sides" effect), thus indicating that DA was present for overall wing shape. This finding is contrary to our previous claim from a subset of O_{st} isochromosomal lines, where DA for some landmarks (e.g. those defining the position of the anterior crossvein) but not for overall wing shape was detected [38]. After plotting the Procrustes grand mean shapes of both wings it also became apparent here that the location of the anterior crossvein was indeed slightly more distal in the right wings. Furthermore, the individuals × sides interaction effects were highly significant in all cases and, hence, wing shape FA greatly exceeded measurement error.

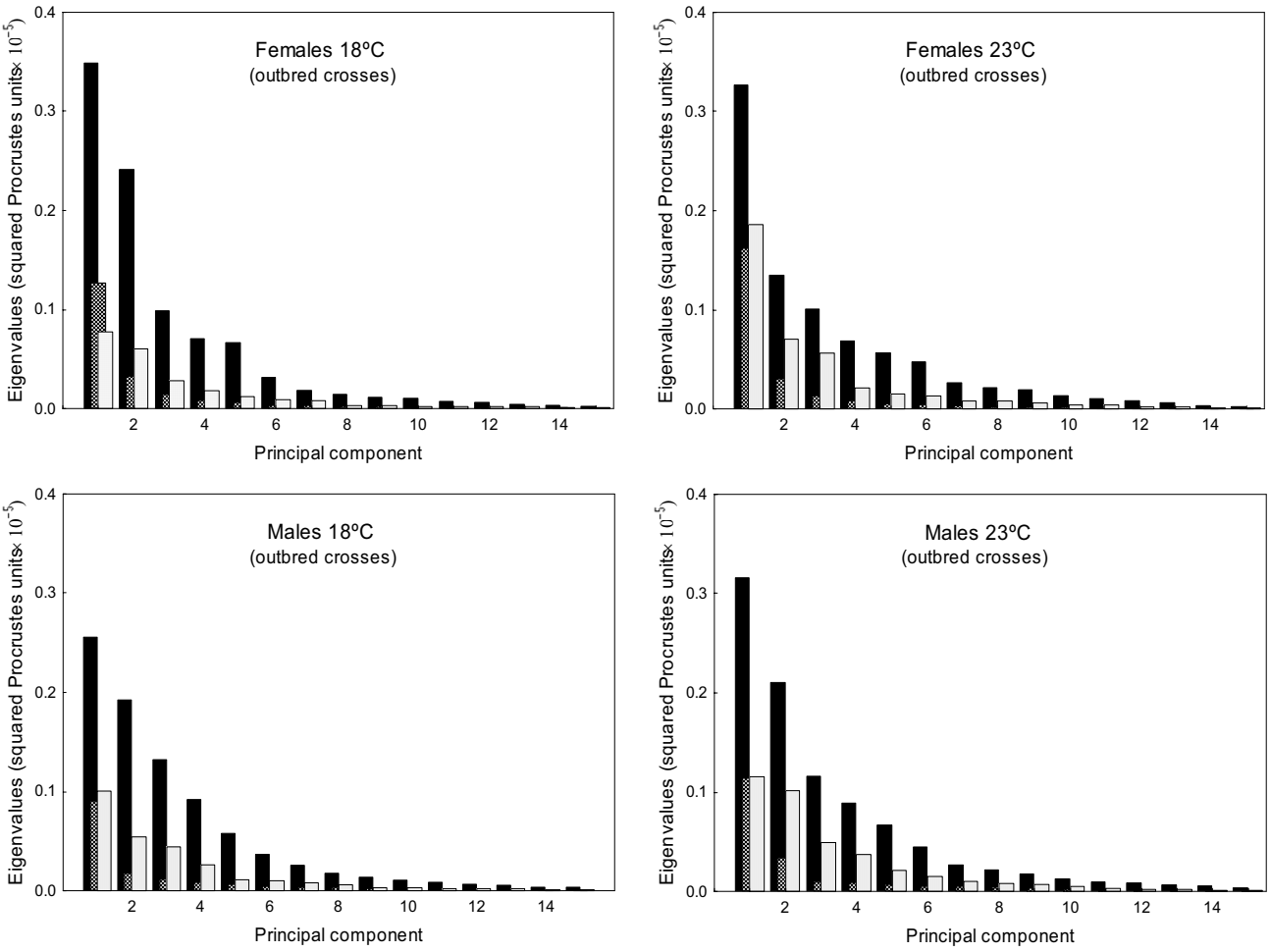

Figure 2
Eigenvalues of causal covariance matrices for wing shape First 15 eigenvalues of the phenotypic (black bars), karyotype (hatched) and crosses (open) covariance matrices from outbred crosses.

b) Causal components of variation

As has been forcefully stressed [42] shape is an inherently multidimensional concept and cannot be easily reduced to a scalar index without severe loss of information. Therefore, for a quantitative genetic analysis of shape data a multivariate approach is required [43]. For overall wing shape, genetic differences among karyotypes were mostly detected for outbred crosses (Tables 5, 6, 7, 8), and we have estimated the covariance matrices **P** = **K** + **C** + **E** as a simple multivariate extension of the two-level nested ANOVAs, where **P** is the phenotypic covariance matrix and **K**, **C**, and **E** are, respectively, the covariance matrices for karyotypes, crosses within karyotypes, and the residuals.

Fig. 2 shows the amount of variation associated with the different dimensions in shape space. Much of the variation was concentrated in the first few PCs, but the **K** matrices showed the clearest trend to quickly decrease after the first PC. Permutation tests indicated that matrix correlations (MCs) between **K** and **C** matrices were generally higher at 18°C (females MC = 0.258, P = 0.1908 ; males MC = 0.305, P = 0.1963) than at 23°C (females MC = 0.157, P = 0.3356 ; males MC = 0.250, P = 0.2665), but none of the MCs was statistically significant. On the other hand, VCV matrices were correlated across rearing temperatures (females: MC **K** = 0.716, P = 0.0163; MC **C** = 0.818, P = 0.0001; males: MC **K** = 0.706, P = 0.0160 ; MC **C** = 0.587, P = 0.0399 ; this last correlation was no longer sig-

Table 9: MANOVAs for female wing shape fluctuating asymmetry A multivariate equivalent of FA1 (i.e., the "unsigned" left-right differences) was defined as explained in the text. Flies raised from outbred crosses of *Drosophila subobscura* (⊂ means 'nested in').

	18°C				23°C			
Source of variation	Wilks' lambda	df 1	df 2	*P*	Wilks' lambda	df 1	df 2	*P*
Karyotypes (K)	0.008	110	48	0.908	0.007	110	48	0.856
Cross ⊂ K	0.022	660	2932	0.169	0.029	660	2932	0.604

nificant after the Bonferroni procedure [44]). A close inspection to Fig. 2 reveals an increase in the genetic components of overall wing shape at 23°C, which agrees with our preliminary findings [38]. Thus, the ratio between the total variance of genetic (**G** = **K** + **C**) covariance matrix onto the total variance of the phenotypic covariance matrix was lower at 18°C in both sexes (females: 0.1312 *vs*. 0.5450; males: 0.2365 *vs*. 0.2522). A caveat: these ratios cannot be interpreted as estimates of shape heritability [43].

MANOVA results in Tables 5, 6, 7, 8 also point to the presence of genetic variation for overall shape DA, mainly at 18°C (i.e., the "crosses in karyotypes" component from the decomposition of the I × S interaction effect). As far as we are aware, these are the first experiments that found detectable genetic variation in DA for wing traits. The uncovering of DA (i.e., "side" effect) for fly wings is quite general when quantitative analyses of form are carried out using the powerful methods of geometric morphometrics to reveal even small morphological variation that otherwise would remain hidden with less effective techniques [13,45]. This has raised concerns against the conventional wisdom that left and right are not distinguished in *Drosophila* development [46] because it provides compelling evidence that DA in fly wings may signal the presence of genetic variation in a phylogenetic conserved left-right developmental axis (i.e., an imaginary plane between the two lateral sides of the body), as discussed by Klingenberg et al. [45]. Actually, modern treatises in developmental biology (e.g. [9]) distinguish the left-right axis besides the customary anterior-posterior and dorsal-ventral axes, and several asymmetrically expressed genes (e.g. *sonic hedgehog*) have recently been discovered. In *Drosophila*, Ligoxygakis et al. [47] were the first (and to our knowledge the only ones) who showed a developmental mechanism for the developmental asymmetry. It seems, therefore, that the detection of genetic variation for DA in this genus appears to be basically a methodological problem, including statistical power and the environmental conditions where the experiments are performed. The mechanisms that constitute the genetic basis of morphological asymmetry in *Drosophila* obviously require further study.

c) Genetic components of wing shape FA

Following [13] a multivariate equivalent of FA1 (i.e., the "unsigned" left-right differences) was defined by changing the signs of all coordinate differences (from left-right to right-left) whenever the inner product (also referred to as the dot product) of a left-right difference vector with the vector of mean left-right difference was negative. For the univariate case (CS) this procedure would render here the absolute ($\bar{L} - \bar{R}$) differences, but notice that for the multivariate case it is not equivalent to calculate the absolute ($\bar{L} - \bar{R}$) differences of all Procrustes coordinates.

MANOVA analyses of these "unsigned" shape asymmetries in outbred crosses did not detect any genetic variation for shape FA at 18°C or 23°C (Tables 9, 10). However, the approach used to define the multivariate equivalent of FA1 might be influenced by the arbitrary choice of the plane (i.e., the mean left-right differences) to subdivide the shape space into "positive" and "negative" halves (Christian P Klingenberg, pers. comm. 2004). A modified Procrustes shape distance for non-isotropic variation (i.e., landmarks usually differ in their amounts of variation) has been recently developed by Klingenberg and Monteiro [48], and can be used here as a scalar measure of the amount of shape asymmetry because FA is random in origin (i.e., only the magnitude and not the direction may usually be the interesting component of FA shape variation). When this scalar was used in our data set the same conclusion was obtained; namely, there was no detectable genetic variation for wing shape FA in any case (results not shown).

d) Consanguinity and temperature effects on wing shape

To investigate allometric and nonallometric temperature effects on overall wing shape we performed a multivariate analyses of covariance (MANCOVA) of the Procrustes coordinates (after averaging both sides and the two replicated measurements per side) considering temperature and inbreeding (i.e., isogenic *vs*. outbred homokaryotypic flies) as the categorical predictors and CS (as $\log_e$ (pixels)) as the covariate. Temperature effects were only significant in males, but inbreeding and temperature × inbreeding interaction effects were highly significant in both sexes

Table 10: MANOVAs for male wing shape fluctuating asymmetry Same as in Table 9.

	18°C				23°C			
Source of variation	Wilks' lambda	df 1	df 2	*P*	Wilks' lambda	df 1	df 2	*P*
Karyotypes (K)	0.004	110	48	0.627	0.009	110	48	0.938
Cross ⊂ K	0.024	660	2932	0.243	0.042	660	2932	0.988

(results not shown), which suggests a strong effect of the categorical predictors on the nonallometric component of shape. Size effects were also found to be significant (females: Wilks' λ = 0.881, $F_{(22,405)}$ = 2.496, P < 0.001; males: Wilks' λ = 0.915, $F_{(22,405)}$ = 1.715, P = 0.024), but the allometric effect on shape remained relatively consistent at both temperatures in females (size × temperature interaction: Wilks' λ = 0.930, $F_{(22,405)}$ = 1.395, P = 0.111) but not in males (Wilks' λ = 0.853, $F_{(22,405)}$ = 3.165, P <0.001). The association between size and temperature (Fig. 1), measured by the variance inflaction factor (*VIF* < 5; [49]), was found to be lower than the suggested guideline for serious collinearity (i.e. *VIF* ≥ 10), which indicates that the effects of temperature and size on wing shape could be effectively separated.

The conclusion is, therefore, that *Drosophila* wing shape does not seem to be as resistant to environmental temperature as previously claimed from the analysis of 12 highly inbred *D. melanogaster* lines [29].

Inbreeding effects (isogenic *vs*. outbred homokaryotypic flies) on wing shape FA were tested from the ratio between the traces of the corresponding "individual × side" VCV matrices. Notice that the traces of these interaction matrices are equal to the respective mean squares of the Procrustes ANOVA as implemented by Klingenberg and McIntyre [13], and are simply the sum of $\mathrm{Var}_{(\bar{L}-\bar{R})}$ (index FA4 in [39]) for each x and y coordinates of the corresponding aligned configurations divided by the shape dimension. We performed 10,000 randomization runs for each test. Inbreeding effects were detected at 18°C but only in females (18°C: female F = 1.694, P = 0.0003 ; male F = 0.963, P = 0.6037 ; 23°C: female F = 0.834, P = 0.9231; male F = 0.984, P = 0.5541).

Patterns of wing shape variation

a) Fluctuating asymmetry

Principal component analyses were only implemented for the outbred crosses since they are more representative of the natural situation. The percentages of total shape variation, together with the features of variation associated with the dominant PCs, are graphically plotted in Figs. 3, 4, 5, 6. For the individual variation several PCs accounted for relatively large amounts of variability. On the contrary, for FA and measurement error PC1 explained almost all total variance (>80%). For all levels in the analysis (i.e. individuals, FA and measurement error) the dominant PCs were connected to the relatively large variability of landmarks 3, 6, 7 and, to a lesser extent, landmark 2. However, the disproportionate amount of variation associated with these landmarks did not spread to all sources of causal variation because their coefficients were relatively small for the PC1 of karyotype variation (which explained ~60% of the total variance; see below). Furthermore, for the individual variation the first two PCs were also linked to the shift of the anterior (landmarks 11 and 12) and posterior (landmarks 7 and 13) crossveins along the adjoining longitudinal veins.

Permutation tests indicated that VCV matrices were mostly correlated for FA and measurement error effects within samples (MCs > 0.95, P < 0.01; Table 11). The individual VCV matrix was significantly correlated with the FA and measurement error matrices only for females at 18°C. Between temperatures the VCV matrices were highly correlated for FA and measurement error (results not shown), but loosely correlated for the individual variation (females MC = 0.668, P = 0.0355 ; males MC = 0.494, P = 0.1066 ; statistical significance vanishes after the Bonferroni procedure).

The angles between the PC1s for FA and measurement error were very much alike (ranging from angle α = 2.1° to α = 3.4° ; recall that the 0.1% quantile of the resulting distribution between pairs of random vectors in 22-dimensional space was 50.3°), which reflects the similarity due to landmarks 3, 6 and 7. However, the first three PCs for interindividual variation were generally distinct to those of FA: the only clear correspondences were between the PC1s for females at 18°C (α = 21.5°), and the PC2 of interindividual variation with the PC1 of FA for males at 18°C (α = 11.8°). (The correspondences were qualitatively the same for interindividual variation and measurement error; results not shown.) Overall, these results seem to suggest that canalization and DS do not generally share the same underlying regulatory mechanisms (but see below).

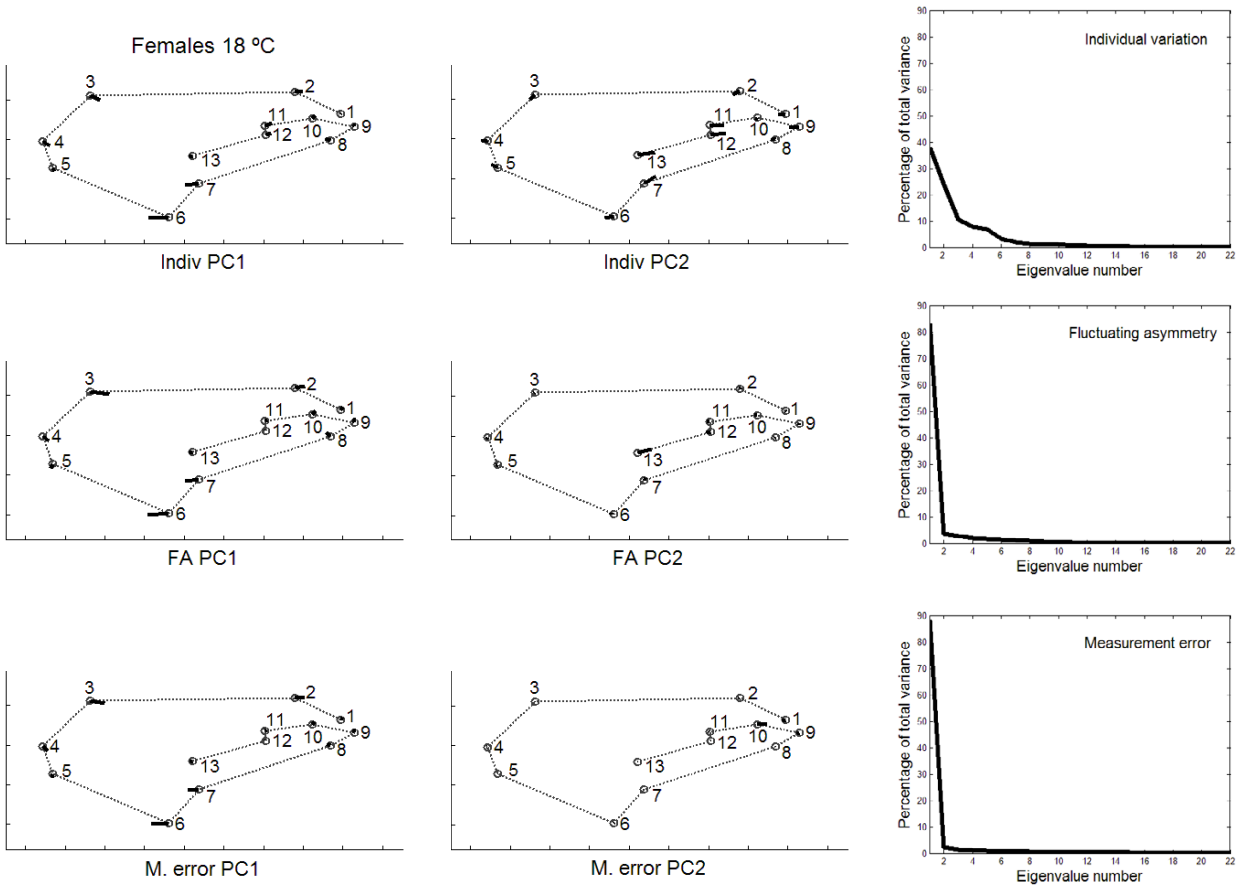

Figure 3
Vectors of the landmarks displacements First two axes of wing shape variation for each effect in the two-way mixed MANOVA (individuals, individuals × sides interaction, and measurement error) for females from outbred crosses reared at 18°C. Also plotted are the percentages of total wing shape variation explained by the principal components for the corresponding covariance matrices.

A potentially important problem with the foregoing approach to compare the patterns of intra- and interindividual variation is to rely on the interaction VCV matrix as the source of variation due to FA. As has been previously argued the uncovering of DA is almost ubiquitous for shape data when using the methods of geometry morphometrics, and there was evidence here for statistically significant genetic variation of overall shape DA at 18°C (Tables 5, 6). Therefore, the VCV matrix from the "individuals × sides" interaction effect gives a biased estimate of developmental stability and cannot be taken as the covariance matrix for FA. In other words, this VCV matrix also includes all causal components due to genetic variation for DA, and the corresponding unbiased VCV matrix for FA is that for the within-fly component of the interaction effect (i.e., after removing the genetic variation for DA [50,51]). In any case, all results were qualitatively similar and, hence, the conclusion that canalization and DS seem to be different mechanisms remains unchanged. However, it is difficult to appraise how this potential problem could have affected the previously published conclusions when comparing interindividual variation and "FA" in fly wings and mouse skulls (see Background section).

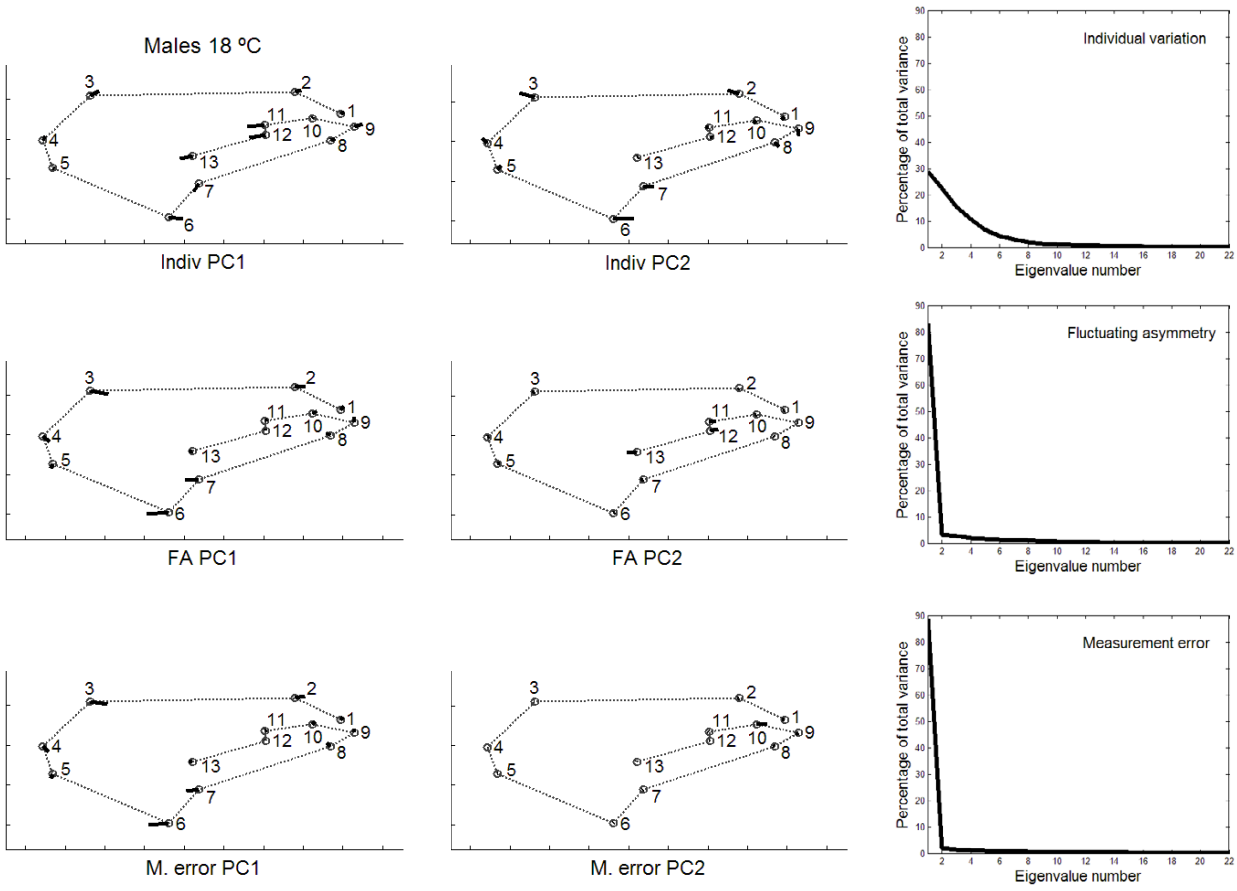

Figure 4
Vectors of the landmarks displacements Same as Fig. 3 for males from outbred crosses reared at 18°C.

Between rearing temperatures the congruence of PC1 eigenvectors was also very high for FA (females $\alpha = 4.0°$; males $\alpha = 3.5°$) and measurement error (females $\alpha = 3.1°$; males $\alpha = 4.1°$). For the interindividual variation the correlations between PC1s were significant only in males (females $\alpha = 74.3°$; males $\alpha = 19.3°$); however, the PC1 vector describing the joint interindividual variation of landmark position in females at 18°C matched the PC2 of the interindividual covariance matrix at 23°C ($\alpha = 49.6°$; recall that the direction of PCs is arbitrary and all the movements in Figs. 3, 4, 5, 6 can be simultaneously reversed by 180°) and *vice versa* (i.e., PC1 at 23°C *vs* PC2 at 18°C: $\alpha = 26.4°$).

b) Causal components

Besides the interindividual variation in the two-way MANOVAs (which comprises genetic plus environmental covariances due to special environmental effects) it is important here to asses the patterns of joint displacements of landmarks for each of the causal components of wing shape variation (Figs. 7, 8, 9, 10). For karyotype variation PC1 accounted for ~60% of the total variance and was linked to a great extent with equivalent movements of those landmarks defining the location of the crossveins, which shifted in the same direction. Landmarks 4 and 5 tended to move away each other, stretching the wing margin between longitudinal veins III and IV. Landmark 9 budged in the opposite direction to crossveins shifts, thus shaping the relationship between L1 to

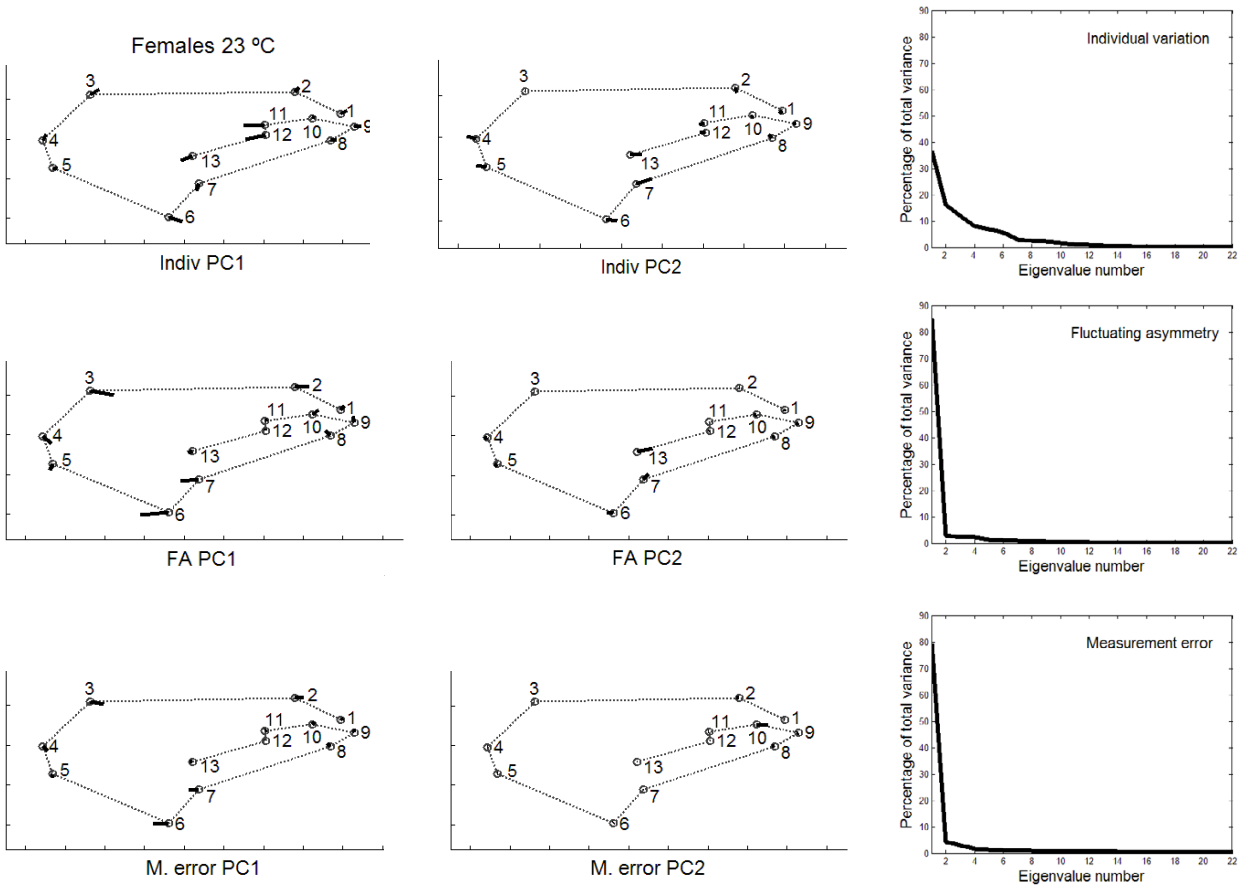

Figure 5
Vectors of the landmarks displacements Same as Fig. 3 for females from outbred crosses reared at 23°C.

the total length of longitudinal vein IV (i.e. shape index L1 WL ; Fig. 12).

A relative shortening of the basal length of longitudinal vein IV relative to the total wing length with an increasing dose of standard gene arrangements in all five major chromosomes of *D. subobscura* had been previously identified in an outbred stock [32,33]. A similar pattern regarding O_{st} dose is also clear here when considering the six karyotypes (Fig. 11), but rearing temperature quantitatively modified the shape index (L1/WL was lower at the highest temperature). However, there was no statistically significant karyotype × temperature interaction. The wing shape index appears to be a purely additive trait since heterokaryotypes were always intermediate to their corresponding homokaryotypes (Fig. 11). Actually, none of 12 within- group (i.e., sex and temperature) possible contrasts comparing all three heterokaryotypes with the average of the corresponding homokaryotypes was statistically significant (the mean square for "crosses" was used as the error term; see legend in Fig. 11).

PC2 for karyotypes was also connected to the variability of landmarks 3, 6 and 7. For the crosses component, several PCs explained relatively large amounts of variation, and shifts of crossveins now seem to be independent of each other at 18°C but not at 23°C. Finally, for the within-fly variation several PCs accounted for relatively large amounts of variability. PC1s were again connected to the variability of landmarks 3, 6 and 7; and PC2s to shifts in the anterior crossvein.

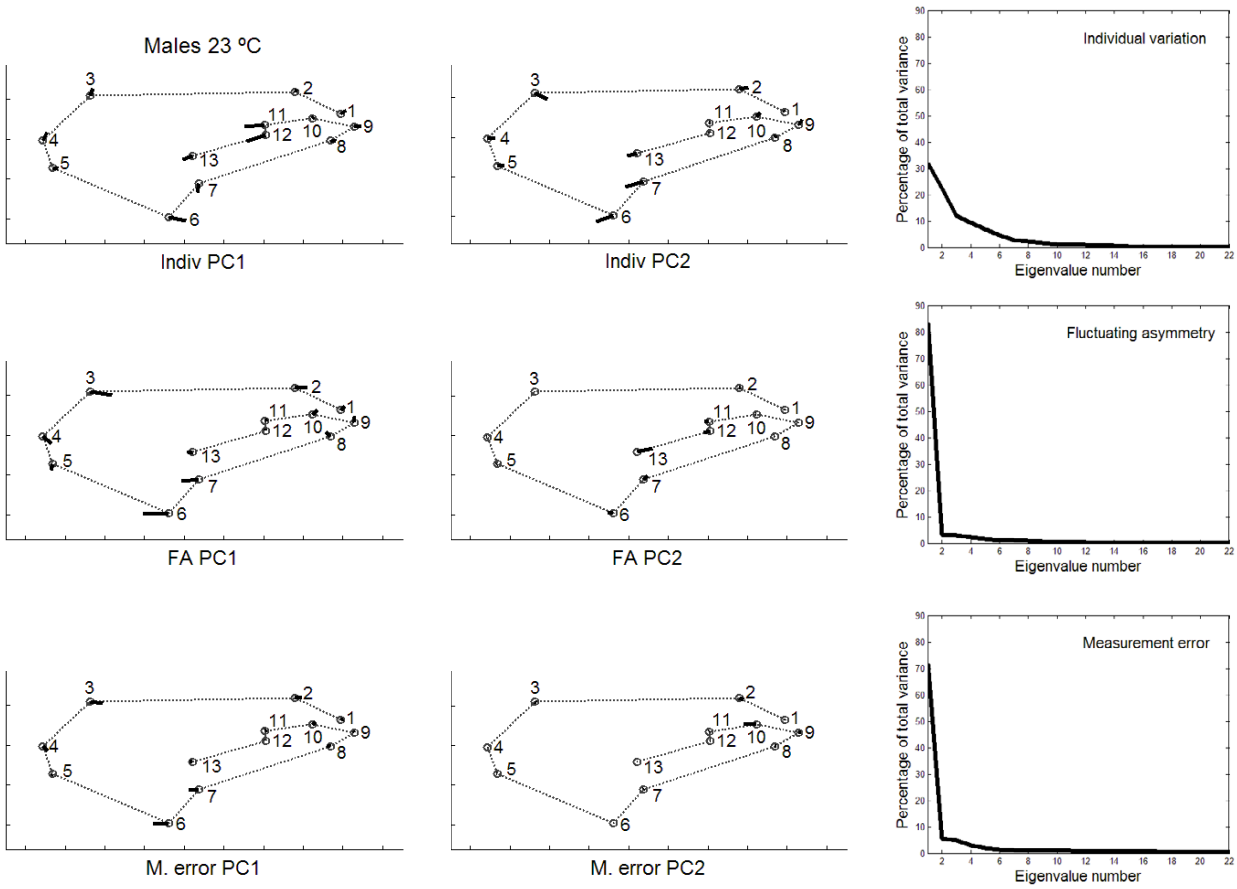

Figure 6
Vectors of the landmarks displacements Same as Fig. 3 for males from outbred crosses reared at 23°C.

The large amount of variation of the anterior and posterior crossveins for karyotypes and crosses can be interpreted in terms of developmental processes. The crossveins are determined after the longitudinal veins, and mutations that eliminate crossveins (e.g. *crossveinless*) do not affect the longitudinal veins; however, some mutants that affect the longitudinal veins also influence the crossveins (e.g. the *vn* group in [1]). Intra- and interespecific studies in several *Drosophila* species have found displacements of one or both crossveins along their longitudinal veins, and such shifts also occur in a number of mutants (see [23]). However, these shifts do not occur in isolation an also include other landmarks as well (e.g., landmarks 9 and 5 on L4; landmarks 1 and 2 on L1; Figs. 7, 8, 9, 10).

The matrix permutation tests (Table 11) indicated that the VCV matrices of karyotypes and crosses were never significantly correlated with the VCV matrices of FA and measurement error. The high correlation between the VCV matrices of the interindividual and FA effects for females at 18°C was basically due to the (micro-) environmental component. Also notice that all correlations between the VCV matrices of karyotype and FA effects were close to zero or even negative, which clearly suggests that this genetic component of canalization is unrelated to DS.

In addition, the PC1s of karyotypes and FA were nearly at right angles (18°C: females α = 85.8°, males α = 77.3°; 23°C: females α = 75.6°, males α = 78.5°). The only matches were between PC2 of karyotypes and PC1 of FA

Table 11: Correlations between VCV matrices of landmarks displacements within groups Results of the permutation tests used for the analyses within sexes and temperatures.

Group	Effects	Correlation	*P* (permutation)	*P* (Bonferroni)
Females 18°C	Individual / FA	0.7699	0.0001	**
	Karyotype / FA	-0.1691	0.7583	n.s.
	Cross / FA	0.5773	0.0871	n.s.
	Between-fly / FA	0.7517	0.0001	**
	Individual / error	0.7550	0.0001	**
	FA / error	0.9953	0.0001	**
Males 18°C	Individual / FA	-0.3998	0.8694	n.s.
	Karyotype / FA	0.0067	0.4393	n.s.
	Cross / FA	-0.0706	0.6202	n.s.
	Between-fly / FA	0.2060	0.3296	n.s.
	Individual / error	-0.4280	0.9436	n.s.
	FA / error	0.9964	0.0001	**
Females 23°C	Individual / FA	0.1233	0.2881	n.s.
	Karyotype / FA	-0.0151	0.4771	n.s.
	Cross / FA	0.6516	0.0264	n.s.
	Between-fly / FA	0.5764	0.0744	n.s.
	Individual / error	0.1093	0.3141	n.s.
	FA / error	0.9959	0.0001	**
Males 23°C	Individual / FA	0.5278	0.0523	n.s.
	Karyotype / FA	-0.1469	0.7545	n.s.
	Cross / FA	0.2752	0.1817	n.s.
	Between-fly / FA	0.4033	0.1241	n.s.
	Individual / error	0.5165	0.0519	n.s.
	FA / error	0.9922	0.0001	**

n.s. = $P > 0.05$; ** = $P < 0.01$.

for females at 18°C (α = 13.0°) and males at 23°C (α = 31.2°). The PC1s of crosses and FA were also poorly correlated; the only exception being females a 23°C (α = 43.3°). These results clearly support the hypothesis that genetic canalization and DS are not functionally the same mechanism.

On the other hand, all observed angles involving PC1s between "replicated genotypes" (i.e. the between-fly component) and FA were relatively small and highly significant (18°C: females α = 20.1°, males α = 15.9° ; 23°C: females α = 22.7°, males α = 36.7°). (Results were qualitatively the same for all observed angles involving PC1s of the between-fly and measurement error covariance matrices; results not shown.) Together with the overall comparisons of the covariance matrices (Table 11), these results indicate that (micro-) environmental canalization and DS share underlying regulatory mechanisms but are not identical. There was not a complete congruence as PC1 of FA accounted for most part of the variation, while PC1 of between-fly variation usually explained less than 50% of the total variance (Figs. 7, 8, 9, 10).

To conclude, the theoretical lower limit for (micro-) environmental canalization (i.e., the environmental variance among genetically identical individuals) would be FA because the two sides share the same genome (barring unusual somatic mutation or somatic recombination) and nearly the same environment, so differences between sides are likely to be small. Under stabilizing selection this lower limit is obviously associated with higher fitness. However, this "canalization limit" would hardly ever be observed because of unavoidable additional sources of environmental variance (e.g. variation between vials, the position of the pupae in a vial, etc.). A similar logic than the one used in this work has been applied to distinguish between intrinsic and extrinsic stochastic variation in gene expression: intrinsic noise can be separated by contrasting the levels of gene expression in a construct with two identically regulated but fluorescently distinguishable *gpf* genes in the *Escherichia coli* chromosome, whereas extrinsic noise is inferred by the correlated variation between the two copies in the same environment [52,53].

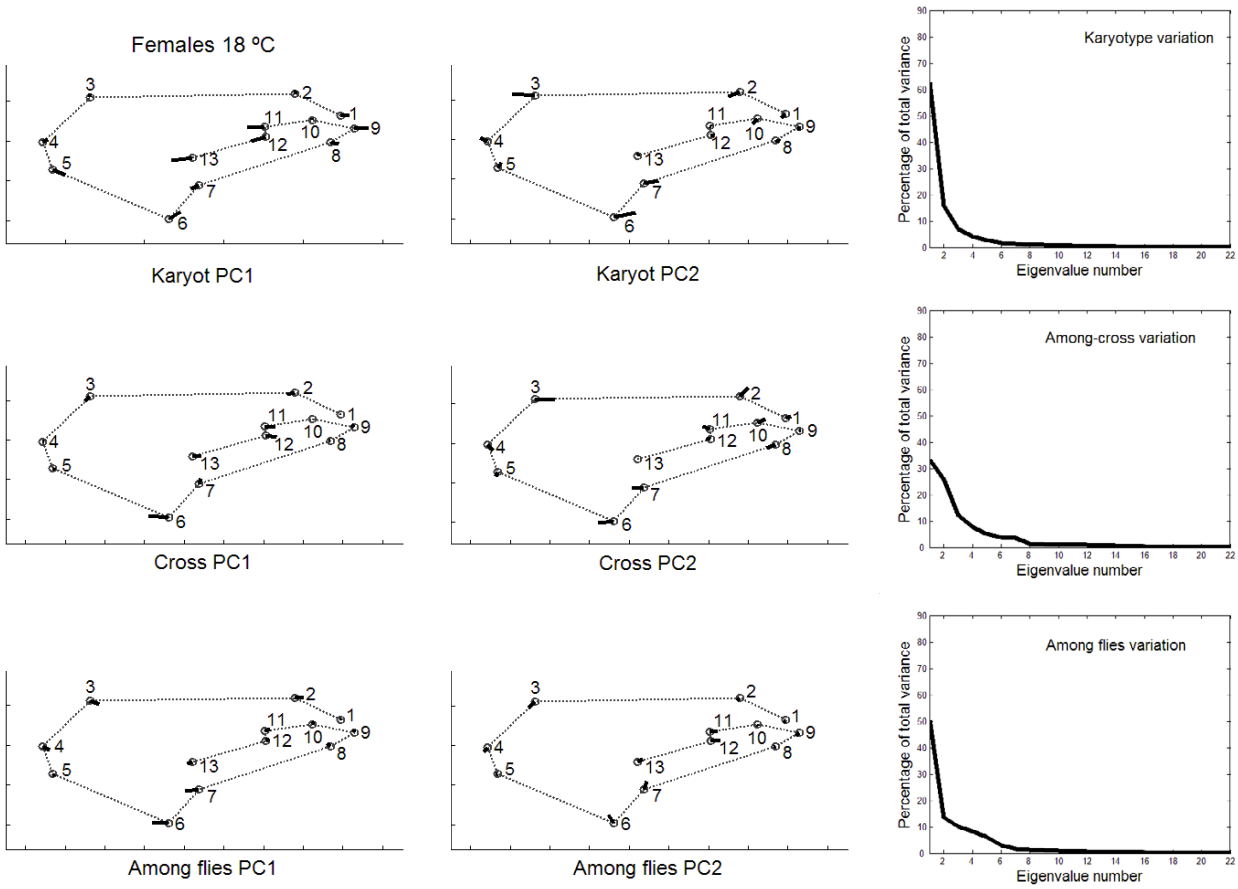

Figure 7
Vectors of the landmarks displacements First two axes of wing shape variation in the two-level nested MANOVA (karyotypes, crosses nested in karyotypes, and within crosses) for each causal component effect pertaining to the inter-individual variation in females from outbred crosses reared at 18°C. Also plotted are the percentages of total wing shape variation explained by the principal components for the corresponding covariance matrices.

Conclusions

This study applied the methods of geometric morphometrics in the context of quantitative genetics of wing form variation using isochromosomal lines of *D. subobscura*. The main findings can be summarized as follows: (i) for the analysis of overall size, DS was positively correlated with levels of heterozygosity (i.e., inbred *vs*. outbred homokaryotypes) and development at the optimal temperature; however, no positive association was found between DS and chromosomal heterozygosity in outbred crosses; (ii) there was detectable genetic variation (mainly for overall shape) for the directional component of morphological asymmetry (i.e., DA) but not for FA, which likely reflects variation due to stochasticity in development; (iii) for analyses of shape, the patterns of covariation for FA and measurement error were highly concordant in all samples, which also provides strong reasons to conclude that FA is generated by random perturbations of developmental processes (obviously, this does not imply that DS is independent of the genetic background: wing shape FA was found to be higher in inbred females at 18°C when compared to their outbred homokaryotypic counterparts); (iv) the inter- and intraindividual variation patterns were generally poorly correlated, which supports the hypothesis that canalization and DS are distinct mechanisms; however, (v) the

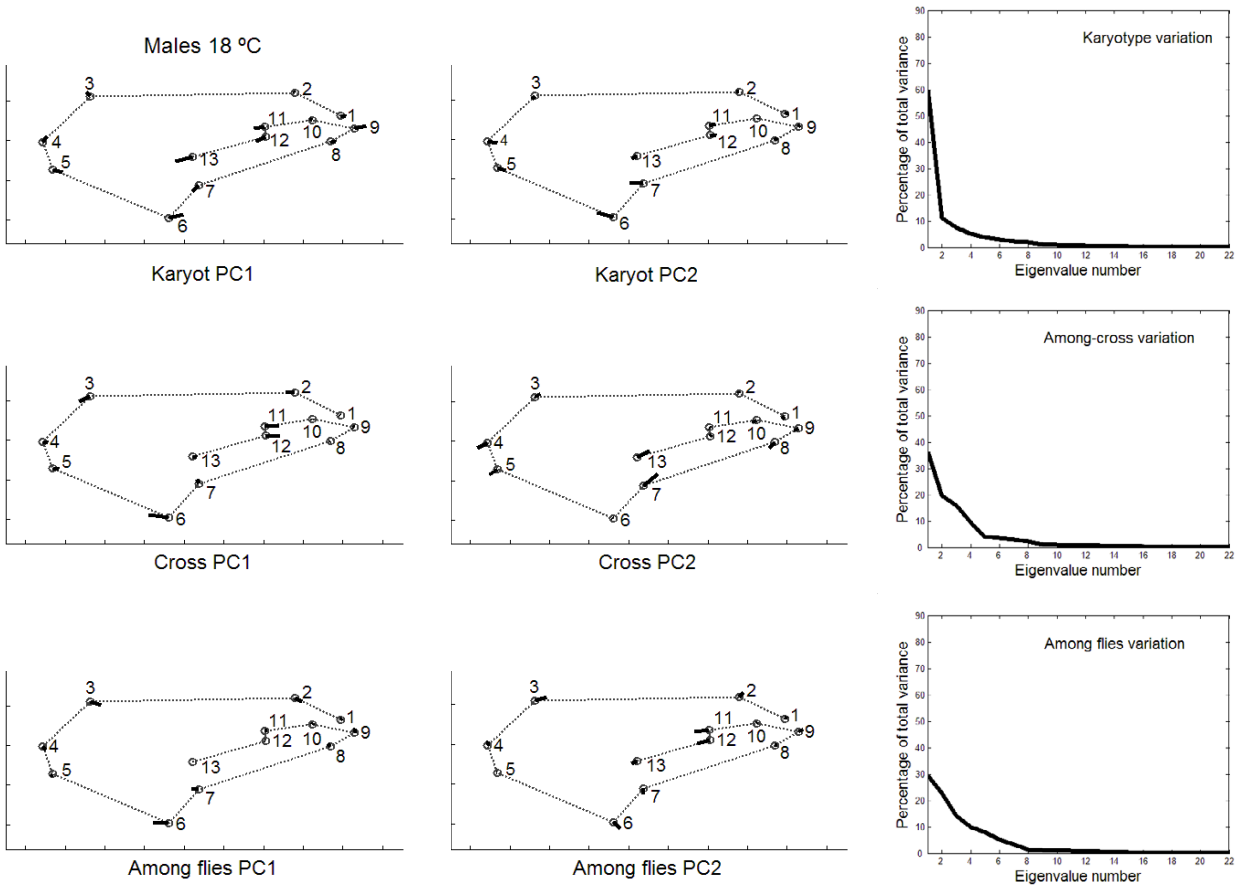

Figure 8
Vectors of the landmarks displacements Same as Fig. 7 for males from outbred crosses reared at 18°C.

patterns of variation due to the (micro-) environmental component of canalization (i.e., the among-fly special environmental effects covariances) were quite similar to those observed for FA; (vi) the lack of a significant within-group correlation between the VCV matrices associated with the interindividual genetic components of canalization and FA, as well as the low similarity between the corresponding vectors describing variation of landmark position, strongly suggest that genetic and environmental canalization are not similar mechanisms.

In addition, (vii) a discrepancy between sexes was observed in some situations; e.g. overall size FA increased with inbreeding and (sub-optimal) temperature effects mainly in females, and the allometric effect on wing shape at both experimental temperatures was similar in females but not in males. It is also interesting to note here that wing size (measured as WL; Fig. 12) clines for *D. subobscura* developed in North America after ~20 years since colonization, but males were clearly lagging behind females [54]. What is not obvious, however, is why there is a difference between the sexes.

It has been suggested that a relationship between canalization and DS could only reflect a common underlying association between character and fitness [55,56]. Those traits under strong stabilizing selection may not be genetically canalized and the major source of selective pressure for canalization can result from the benefits gained by buffering the effects of environmental perturbations

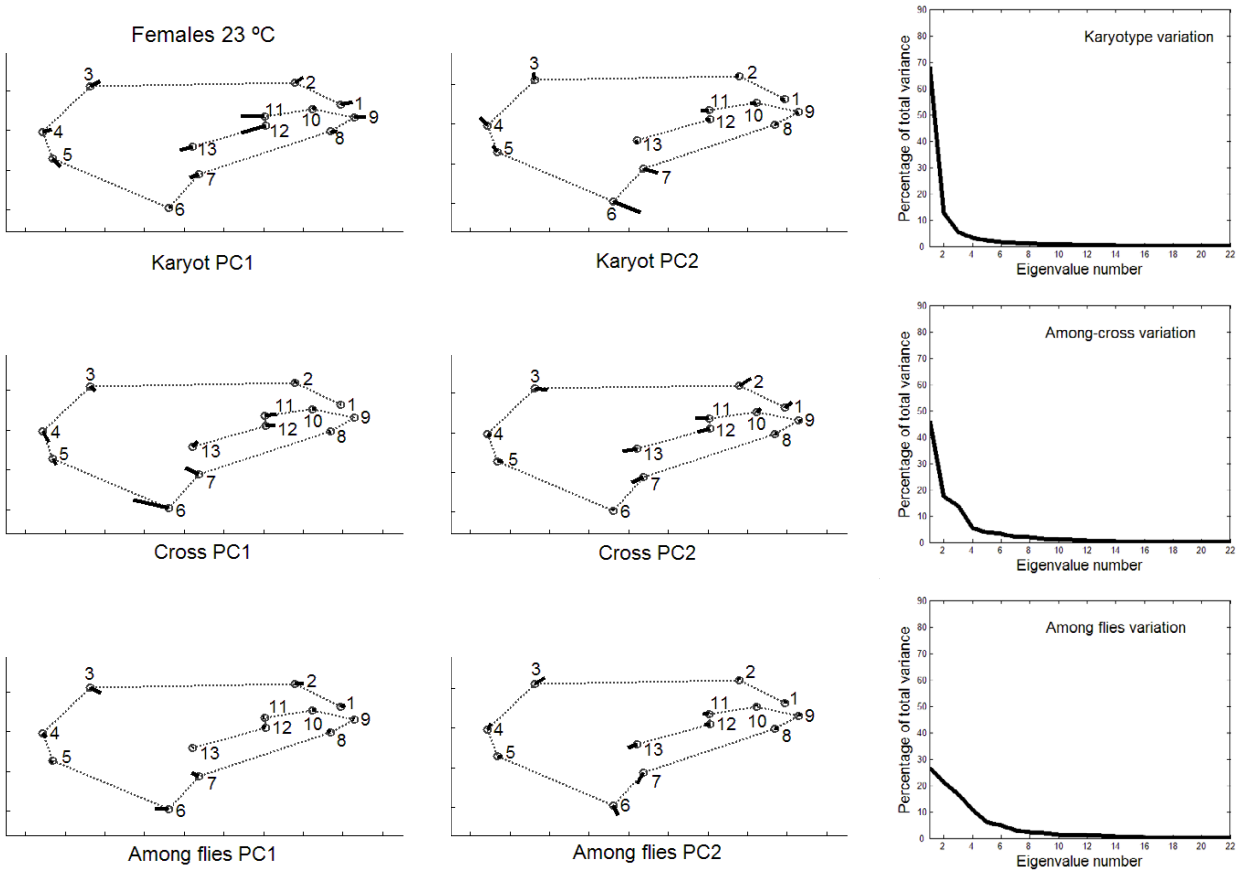

Figure 9
Vectors of the landmarks displacements Same as Fig. 7 for females from outbred crosses reared at 23°C.

[4,10]. The strongest evidence in favor of this hypothesis comes from the well-known genotype-phenotype mapping of RNA folding. Conservation of RNA secondary structure is under strong selection, and low structural plasticity is achieved through increasing the thermodynamic independence of any one structural component from the remaining structure [57]. Likewise, the flux summation theorem developed in the field of metabolic control analysis implies, if true, that phenotypic robustness is an inevitable outcome of the underlying metabolism and not a result of evolution (see [58]).

However, it is still an open question whether or not natural wing shape changes in *Drosophila* are adaptive. There are no consistent patterns between latitude and wing shape (e.g. [30]), contrarily to what happens for size-related traits where world-wide latitudinal clines are found with genetically larger individuals derived from higher latitudes (e.g. [30,59]). Many genes with small additive effects on features of wing shape are dispersed along the *Drosophila* genome (e.g. [60,61]), and we have shown here that the wing shape index L1/WL appears to be a purely additive trait since heterokaryotypes were always intermediate to their homokaryotypic counterparts. The wing shape cline in North America colonizing populations of *D. subobscura* [30] can be largely accounted for parallel latitudinal clines in chromosomal gene arrangements [32,33], and the small shifts of (e.g.) the anterior and posterior crossveins in relation to karyotype variation (Figs. 7, 8, 9, 10; notice that the plotted joint var-

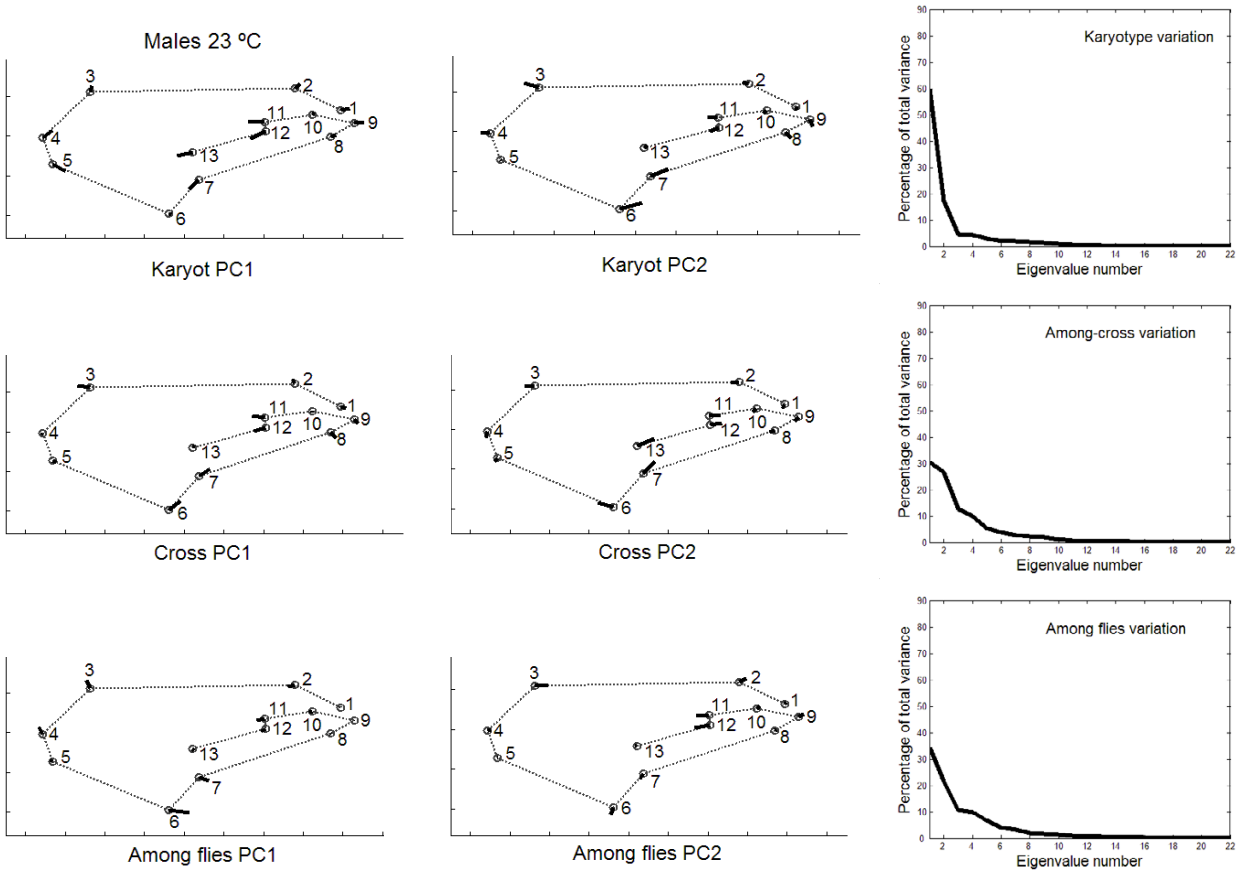

Figure 10
Vectors of the landmarks displacements Same as Fig. 7 for males from outbred crosses reared at 23°C.

iation in landmark positions is an exaggeration of the actual variation in the data set) are difficult to link with any adaptive response to a better flight capacity. Actually, we lack even hypothetical functional explanations for subtle shape variation: Gilchrist et al. [54] speculated that wing shape variation in *D. subobscura* may simply represent drift around an optimum. Our present results (points (v) and (vi) above) give some credence to that conjecture. Genetic canalization on wing shape does not seem to arise as a by-product of environmental canalization and, therefore, canalization is not a single mechanism to buffer any source of variation as has been suggested [10].

According to Graham et al. [62] the classical linear theory of DS can successfully account for both normally distributed error distributions and leptokurtic distributions caused by the admixture of individuals having different levels of DS, but cannot account for transitions between FA and DA. We have previously suggested, however, that a transition from "ideal" FA (i.e., a normal distribution of left – right-side scores whose mean is zero) to a distribution showing DA could be made entirely compatible with what it is already known from classical quantitative genetics [38]. Shifts between asymmetry types (FA, DA and antisymmetry) have been reported to happen along a species distribution range [63], but unless the genetic component can be partitioned out the variation in left-right differences cannot be assumed to describe DS. From the results of outbred crosses reared at 18°C (Table 5, 6) it is possible to test here for the congruence between pat-

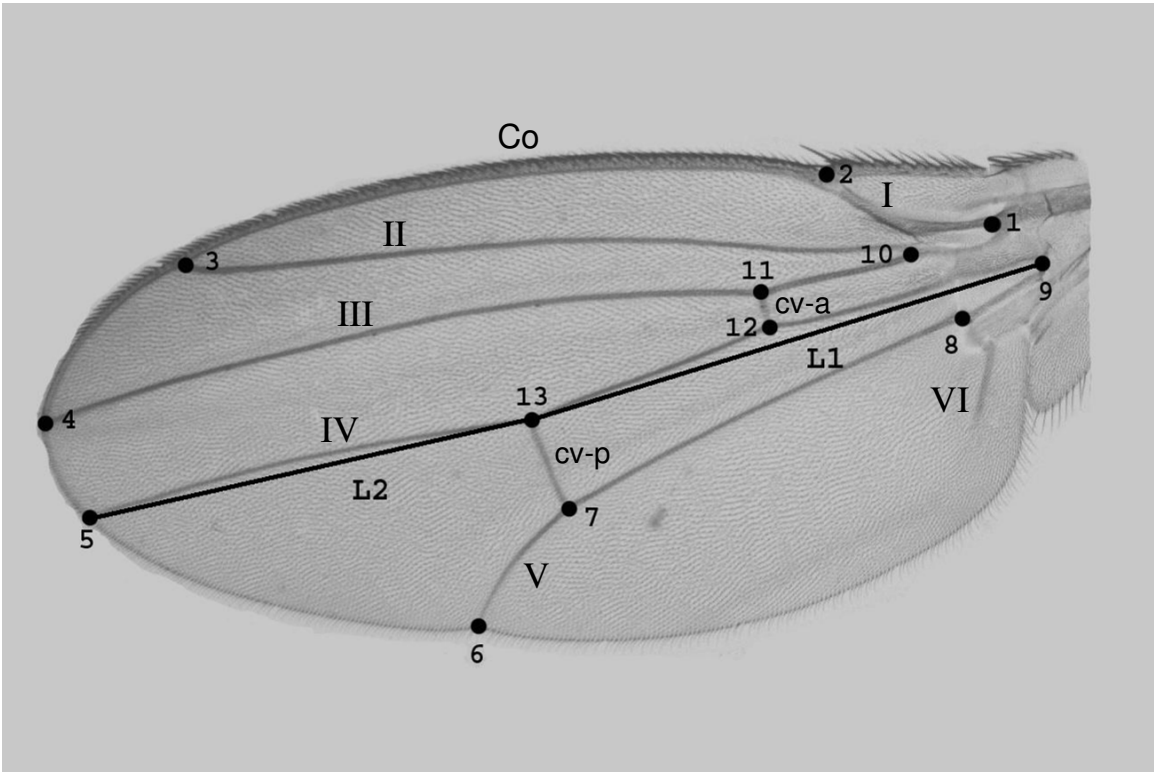

Figure 12
Left wing of *Drosophila subobscura* The image shows the thirteen landmarks (1 – 13) used in this work. I – VI longitudinal veins; cv-a and cv-p anterior and posterior crossveins; Co costal or marginal veins; L1 and L2 lengths of the proximal (Euclidian distance between landmarks 9 and 13) and distal (Euclidian distance between landmarks 13 and 5) segments of longitudinal vein IV, respectively. Wing shape index $L1/WL$ has been previously used to study shape clines in this species [30].

terns of morphological variation with respect to the variation attributable to FA (i.e. the within-fly environmental component of the interaction term) and that attributable to genetic variation for DA (the within-fly genetic component due to crosses in karyotypes of the interaction term). The corresponding VCV matrices were highly correlated for females (MC = 0.914, $P_{(permutation)}$ = 0.0001) but not for males (MC = 0.064, $P_{(permutation)}$ = 0.485). The angles between the PC1s also reflect this discrepancy between samples (females α = 8.6° ; males α = 45.9°). When considered together, these results clearly suggest that FA and genetic variation for DA may or may not be functionally linked.

Methods

Extraction of O chromosomes and fly handling

A large number of *D. subobscura* isochromosomal lines for the O chromosome in an otherwise homogeneous genetic background were derived from an outbred stock collected at Puerto Montt (Chile; 41° 28' S) in November 1999 as previously indicated [38]. Briefly, wild-type males were individually crossed to three or four virgin females from the highly homogeneous *ch-cu* marker strain, which is homozygous for the morphological recessive markers on the O chromosome *cherry* eyes (*ch*) and *curled* wings (*cu*) and fixed for the gene arrangement O_{3+4}. A single O^{1}_{++++}

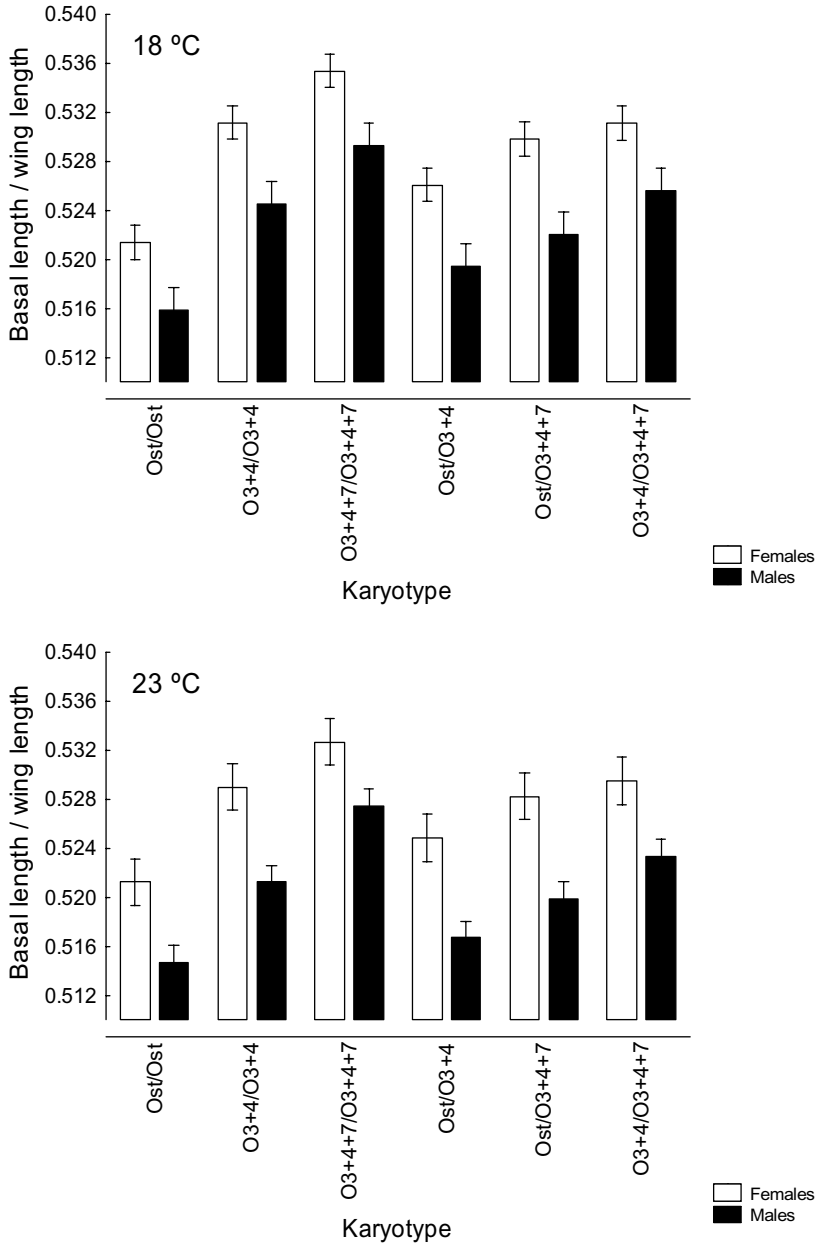

Figure 11

Wing shape index Averages of the relative length (with 95% confidence intervals) of the basal portion of longitudinal vein IV (L1) to the total wing length (WL = L1 + L2) versus karyotype for outbred crosses at the two rearing temperatures. A two-way factorial ANOVA using the shape index as $\log_e\left(\mathrm{L1}/\mathrm{WL}\right)$, with karyotype and temperature as fixed effects, and crosses nested within karyotypes, detected statistically significant differences for the main effects (karyotype: female $F_{5,30} = 12.625$, $P < 0.001$; male $F_{5,30} = 9.785$, $P < 0.001$. Temperature: female $F_{1,390} = 30.219$, $P < 0.001$; male $F_{1,390} = 61.835$, $P < 0.001$) but no karyotype × temperature interaction (females: $F_{5,390} = 1.570$, $P = 0.168$; males: $F_{5,390} = 1.111$, $P = 0.354$).

/ O_{+cu+ch} male from the offspring was backcrossed to *ch-cu* females, and its arrangement on the wild-type chromosome was identified after four generations of backcrosses. Followed by at least another backcross to *ch-cu* females, a single male from each line carrying the wild chromosome was crossed to two virgin females from the *Va*/*Ba* balanced marker stock. This strain was derived from the *ch-cu* stock and carries the dominant lethal genes *Varicose* (*Va*) and *Bare* (*Ba*) on the O chromosome. The isochromosomal lines were established from the final crosses ♀♀ $O_{Va\,ch+cu}$ / O^{1}_{++++} × ♂♂ $O_{Va\,ch+cu}$ / O^{1}_{++++}. All lines used here had a quasi-normal viability according to the recorded proportions of wild-type flies raised in the final crosses to obtain the isochromosomal lines [38]. The lines were kept at 18°C (12:12 light/dark cycle) in 130-mL bottles with low adult density to standardize the rearing conditions before egg collections.

As previously indicated the experimental flies were obtained from 54 crosses. Reciprocal crosses were made for all outbred combinations by mating one-week virgin females and males. After three days the males were discarded and equal numbers of females from each reciprocal cross were placed together in a plastic chamber with a spoon containing non-nutritive agar with a generous smear of live yeast for egg collections. To standardize the experimental conditions, eggs from the inbred (isogenic) crosses were also obtained in a similar way; namely, after mating the flies in bottles and transferring the females to plastic chambers. Eggs were placed in six 2 × 8 cm vials with 6 mL of food (26 eggs/vial); three vials were kept at 18°C (optimal temperature) and the other three at 23°C (sub-optimal temperature). Within each experimental temperature the vials were randomly placed on the same incubator shelf. As a result, the total experiment consisted of 324 vials (162 vials at each experimental temperature), and all eggs were sampled on the same day. Emerging flies (not less than 2 or 3 days old) were stored in Eppendorf tubes with a 3:1 mixture of alcohol and glycerol at 4°C before wing measurements.

All fly handling was done at room temperature using CO_2 anesthesia on flies not less than 6 h after eclosion.

Wing measurements

Two randomly sampled females and males emerged from each vial were used for morphometric analyses. Both wings were removed from each fly and fixed in DPX under coverslips on microscope slides. Bitmap images were captured with a video camera (Sony CCD-Iris, Tokyo, Japan) connected to a PC computer with MGI VideoWave software and mounted on a compound microscope (Zeiss Axioskop, Jena, Germany), using a 2.5 × objective. Calibration of the optical system was checked at each session. The images were stored on a Dell Workstation PWS350. To quantify and minimize measurement error all wings were digitized two times at different sessions as follows: images of both the left and right wings were captured during a given session and after an entire round on all individuals the same process was repeated again. A similar procedure was also used to record the *x* and *y* coordinates of 13 morphological landmarks (i.e., labeled geometric points located at the intersections of wing veins or at sites where veins reach the wing margin; Fig. 12) by using the Scion Image for Windows software [64]. Therefore, the process we used guaranteed that the observer was blind with respect to the results from previous measurements.

Analysis of wing size and shape

Geometric morphometrics precisely separates morphological variation (i.e., variation in form) into size and shape components [21,22]. Size is a one-dimensional trait and the measure most widely used in geometric morphometrics is centroid size (CS), computed here in a normalized form as the square root of the sum of squared Euclidian distances between each landmark to the centroid (center of gravity) of all landmarks divided by the square root of the number of landmarks. Individual size is therefore represented by four scalars, one for each side and session.

The shape of an original configuration of landmarks is the geometrical information that is invariant to uniform scaling (variation in size), translation (differences in position), and rotation (differences in orientation). In contrast to size, shape is an inherently multidimensional space and we used Procrustes superimposition to characterize shape variation. This method allows comparing configurations of landmarks by optimally superimposing (according to a least-squares criterion) homologous landmarks in two or more specimens to achieve an overall best fit [65].Because the data set included both left and right wings (i.e., we are dealing with "matching symmetry" [66,67]) our analyses also removed differences due to reflection by changing the sign of the *x* coordinate of every landmark for configurations from the right side. The reflection, scaling, and superposition steps were performed for all wings within each cross and temperature simultaneously, which allows contrasting wing shapes between different lines or crosses. The final iteration to minimize the sum of the squared distances between the landmarks of all wings in the sample was done without additional scaling and, consequently, we performed a partial Procrustes fit according to Dryden and Mardia [22]. Given the small amounts of shape variation in this analysis rescaling the coordinates of each configuration by the scaling option $1/\cos(\rho)$ [65] would have negligible effects on the results.

The landmark coordinates after Procrustes superimposition are amenable to standard multivariate analyses. However, it is important to remember that the removal of size, position (in two dimensions), and orientation reduces the dimensional space to $2p - 4$, where p is the number of landmarks [22]. Thus, for the present study of 13 landmarks, with 2 coordinates each, the shape dimension is 22. Sums of squares and cross-products (SSCP) matrices are therefore not full-ranked, and the degrees of freedom need to be adjusted. There are three alternative ways of avoiding these difficulties [22,67]: (i) to omit, after Procrustes superimposition of the complete configurations, the coordinates of any two landmarks; (ii) to retain 22 PC scores from the covariance matrix of the data set; (iii) to slightly modify the multivariate statistics (see below) by using the Moore-Penrose generalized inverse of the SSCP matrices so they can tolerate singular matrices, and compute the product of nonzero eigenvalues instead of the determinant of SSCP matrices. We have used here the second scheme.

Experimental design and asymmetry analysis

Quantitative genetic studies of directional and fluctuating asymmetry obviously require measures from individuals that can be grouped into families or independent lines. Our final data set was a fully balanced design, comprising 54 crosses × 3 vials per cross × 2 females per vial × 2 males per vial × 2 sides per fly × 2 measurements per wing × 2 temperatures = 5,184 wing landmark configurations in total. Within each sex and temperature, least-squares (ANOVA) estimates of variance components (i.e. CS) can be easily obtained from the linear model:

$$Y_{ijkl} = \mu + \kappa_i + l_{j(i)} + \nu_{k(ji)} + \varepsilon_{l(kji)},$$

where μ is the overall grand mean, κ_i is the effect of the ith karyotype, $l_{j(i)}$ is the random effect of the jth cross within karyotype i, $\nu_{k(ji)}$ is the random effect of the kth vial within cross j and karyotype i, and $\varepsilon_{l(kji)}$ is the residual error associated with the trait (i.e. the individual means computed from both sides and the two replicated measurements per side) of the lth individual within vial k, cross j, and karyotype i. Since there was no genetic variation within crosses, the residual error provides an estimate of the total special environmental effects variance (i.e. σ_e^2). Variation among the three replicated vials was generally negligible (results not shown) and, therefore, we have conveniently reduced the previous model to a two-level nested ANOVA after grouping flies across vials.

To first partition the total phenotypic variation into interindividual, intraindividual and measurement error components, we used the conventional mixed model, two-way ANOVA (or its MANOVA generalization; see below) for the study of left-right asymmetries [39]. In this ANOVA the main random effect of individuals stands for phenotypic variation in the trait (i.e. CS), the main fixed effect of body side is for directional asymmetry (DA) and tests whether or no the signed differences between the left and right wings [designated as $(\bar{L} - \bar{R})$] have a mean of zero, the interaction term is a measure of fluctuating asymmetry (the variation in left-right differences among individuals) provided that there is no genetic variation for DA [51], and the error term gives an estimate of the measurement error. The two-level nested ANOVA can be straightforwardly subsumed within the two-way ANOVA.

We now digress slightly to point out some inconsistencies in the literature on what is the appropriate error term to test for the "interindividual" effect in the mixed model, two-way ANOVA (either the individual × side interaction effect or the measurement error [13,15,68,69]). Interindividual variation, even if of no general interest in most studies of asymmetry, comprises here genetic components ("karyotype" plus "crosses within karyotypes") and special environmental effects variance (σ_e^2; there is no genetic variance within crosses). An estimated of the among-fly special environmental effects variance (i.e. σ_{ea}^2) is therefore obtained by subtracting the individual × side interaction effect (which includes σ_{ew}^2 plus measurement error) as the appropriate error term. However, when genetic variation for DA is present the unbiased within-fly special environmental effects variance (i.e. FA) is estimated after partitioning the individual × side interaction effect into its causal components [51].

As pointed out by Klingenberg et al. [67] it is fairly straightforward to extent the preceding ANOVA approach to a full two-factor MANOVA to analyze wing shape asymmetry since all effects are computed from averages or contrasts in the same shape space. Recall that the traces of the corresponding SSCP matrices are just the sum of squares in the Procrustes ANOVA as implemented by Klingenberg and McIntyre [13], but this ANOVA is based on an isotropic model (i.e., it assumes that there is an equal amount of non-directional variation at each landmark [70]) that is not generally correct for any real data. Covariance (VCV) matrices for each effect in the MANOVA were calculated as a simple multivariate extension of the two-way ANOVA. Thus, the SSCP matrices were divided by the appropriate degrees of freedom, and effects were separated according to the expected mean squares in the ANOVA by subtracting the interaction covariance (VCV) matrix from the interindividual VCV matrix, and the error VCV matrix from the interaction one. Therefore, for (e.g.) outbred crosses the interindividual covariance components were calculated as

$\frac{SSCP_I/4730 - SSCP_{IS}/4730}{4}$ and the covariance components of FA as $\frac{SSCP_{IS}/4730 - SSCP_{ME}/9504}{2}$, were $SSCP_I$ is the interindividual SSCP matrix, $SSCP_{IS}$ is the interaction SSCP matrix, and $SSCP_{ME}$ is the measurement error SSCP matrix.

The $SSCP_I$ matrix was further partitioned into among-karyotype $SSCP_K$ matrix, among-cross within karyotype $SSCP_{C\subset K}$ matrix, and the residual $SSCP_e$ matrix corresponding to the special environmental effects. As a result, genetic effects for overall wing shape were separated from special environmental effects according to the expected means squares in the two-level nested ANOVA. Therefore, for (e.g.) outbred crosses the karyotype covariance components were calculated as $VCV_K = \frac{SSCP_K/110 - SSCP_{C\subset K}/660}{144}$ (remind that the entries in the $SSCP_I$ matrix are equal to those computed from individual means times twice the number of independent measurements per wing), the cross covariance components as $VCV_{C\subset K} = \frac{SSCP_{C\subset K}/660 - SSCP_e/3960}{24}$, and the among-fly special environmental effects covariance components as $VCV_{ea} = \frac{SSCP_e/3960 - SSCP_{IS}/4730}{4}$.

Similarly, genetic effects for DA can be investigated after partitioning the $SSCP_{IS}$ matrix into their causal components [51].

Morphological patterns of variation

Within each sex and temperature, principal component analyses [41] of the VCV matrices were performed for each source of variation with the purpose of describing the landmark displacements corresponding to each emerging principal component (PC), and also to test for the congruence of these displacements between effects. This technique extracts new shape variables (PCs) which successively account for the maximal amount of shape variation and contain information on how the variables relate to each other. The PCs form an orthonormal set of vectors (i.e., the inner product $\mathbf{p}_i'\mathbf{p}_j = 0$ for i ≠ j, $\mathbf{p}_i'\mathbf{p}_j = 1$ for i = j; superscript ' denotes transposition) in an n-dimensional space.

Correlations between corresponding VCV matrices were computed from the upper triangular part (diagonal entries were included) since covariance matrices are symmetrical, and statistical significance was assessed using permutation tests designed to maintain the associations between pairs of *x*- and *y*-coordinates (i.e., by permuting pairs of rows and columns [13,15]); otherwise the null hypothesis would imply the complete absence of all geometric structure. The permutation procedure was carried out 10,000 times. Correlative patterns of whole shape variation are difficult to interpret: a significant correlation would suggest a real congruence, but a weak congruence does not imply a significant correlation.

A second test examined the congruence of the landmark displacements corresponding to each emergent PC for the different effects within groups. Because the PCs correspond to directions in the multivariate shape space, correlations can be obtained by angular comparisons of component vectors. Statistical significance of these correlations was then assessed by comparing those observed values to a null distribution of absolute angles between 100,000 pairs of 22-dimensional random vectors [71]. The 0.1% and 0.001% quantiles of the resulting distribution were 50.3° and 41.6°, respectively.

Antisymmetry and allometric effects

The occurrence of antisymmetry (AS: a bimodal distribution of signed $(\bar{L} - \bar{R})$ [39]) for centroid size was investigated within each sample using the Lilliefors (Kolmogorov-Smirnov) test for the composite hypothesis of normality [69]. The independence between size and size FA within each sample was assessed by a linear regression of unsigned $(|\bar{L} - \bar{R}|)$ against mean centroid size $(\bar{L} + \bar{R})/2$.

Scatter plots of left-right differences for each landmark after Procrustes superimposition were visually checked to see whether or not there was evidence for clustering of these vectors that would have argue for the occurrence of AS [13,15]. No indication of AS was detected. Finally, to test for size effects on shape asymmetry within each sample we used multivariate regression of vectors of both signed and "unsigned" shape asymmetries onto mean centroid size [13]. Shape asymmetries were not related to size (*P*-values > 0.10) and, therefore, no size corrections were necessary.

Computer software for statistical analysis

The computer programs used for statistical data analyses were MATLAB (V.6. [72]) together with the collection of tools supplied by the Statistics Toolbox (V.3. [73]). Some helpful functions in morphometrics from the MATLAB

toolboxes Res5 and Res6 developed by R. E. Strauss [74] were also used. Results (e.g., derivation of SSCP matrices) were checked with the statistical software packages STATISTICA V.6 [75] and SPSS V.11 [76].

Authors' contributions

MS conceived the study, carried out extraction of O chromosomes, experimental crosses, egg collections, statistical analyses, and drafted the final manuscript. PFI carried out extraction of O chromosomes, experimental crosses, egg collections, wing measurements, and preliminary statistical analyses and drafts of results. WC read all salivary gland squashes for gene arrangement identification and mounted the wings on microscope slides. All authors read and approved the final manuscript.

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3. DISCUSION

Dentro de este trabajo de Tesis Doctoral se han llevado a cabo varios estudios. Una primera parte con el objetivo de dilucidar el papel de la temperatura en el establecimiento del patrón biológico clinal en las frecuencias del polimorfismo de inversión cromosómico y en el tamaño y la forma del ala en poblaciones experimentales de *D. subobscura*. Para esto, un stock de selección termal fue iniciado a partir de una población ancestral muestreada en Puerto Montt, estimado como el punto de origen de la colonización del Nuevo Mundo; se analizaron los tres caracteres, y los resultados obtenidos fueron contrastados en relación con los patrones encontrados en las poblaciones naturales y los experimentos de selección termal en laboratorio en otras especies de *Drosophila*.

Como segunda parte**,** se realizó un análisis genético cuantitativo en una serie de líneas isocromosómicas de *D. subobscura*, fijadas para los diferentes ordenamientos cromosómicos del cromosoma O, para analizar los efectos de la variación genética clinal (polimorfismo de inversión), homocigosis y temperatura, en el tamaño, forma y asimetría bilateral del ala, usando el método de morfometría geométrica. Los resultados obtenidos se discuten en relación con la evolución de los mecanismos de amortiguamiento (canalización y estabilidad de desarrollo) y el posible valor adaptativo de la variación de la forma natural del ala de *D. subobscura*.

3.1. Evolución termal de tamaño y forma del ala

La presencia de clinas latitudinales para el polimorfismo de inversiones cromosómicas y para el tamaño corporal en las poblaciones americanas de *D. subobscura*, similares a las de las poblaciones originales de la región paleártica, junto a la situación geográfica y climática equivalente en ambas áreas de distribución (América y Europa) (Prevosti *et al*., 1988), hacen pensar que ciertos factores ambientales, principalmente la temperatura, podrían tener un papel importante en el desarrollo de estas clinas latitudinales. Por tanto, esta primera parte del estudio es una forma de acercarnos al entendimiento de las posibles relaciones entre la temperatura y la formación de estas clinas latitudinales.

4.1.1. Diversidad cromosómica

Después de dos años de selección termal en el laboratorio se observa que hay un nivel considerablemente alto de diversidad cromosómica en las poblaciones experimentales, donde todos los ordenamientos génicos del Nuevo Mundo están presentes en la población inicial, y segregan en las poblaciones experimentales del stock de selección termal a distintas

frecuencias, excepto para el ordenamiento cromosómico E_{1+2} que es considerado prácticamente extinto en las poblaciones de Suramérica (Prevosti *et al*., 1985).

Por otro lado, y en contraste con el patrón de distribución geográfica del índice de diversidad del Viejo Mundo, hay una relación negativa entre el índice de diversidad cromosómica de las poblaciones experimentales y el efecto evolutivo de la temperatura, con una tendencia decreciente de la diversidad a medida que aumenta la temperatura. Esto podría interpretarse como una perdida de diversidad cromosómica en respuesta al aumento de temperatura, como fue ya observado en estudios previos de evolución termal (Krimbas 1967; Quintana y Prevosti 1991), y en el cambio de diversidad cromosómica relacionada con el cambio climático actual, en los que se encontraron pérdidas de diversidad para el cromosoma O (Rodríguez-Trelles y Rodríguez 1998) o disminución de las frecuencias de los ordenamientos cromosómicos más frecuentes de las poblaciones del norte, en muestras más recientes, probablemente como respuesta al calentamiento global del planeta (Solé *et al.,* 2002).

Los patrones de distribución geográfica del Viejo Mundo no permiten inferir fácilmente la adaptación real, ya que sólo reflejan procesos históricos. El patrón paleártico, conocido como "central" o "marginal" (Krimbas y Loukas 1980), muestra que el índice de diversidad disminuye en dirección Sureste - Noroeste y con un punto relativamente alto en el noreste de España (Krimbas 1992). Todo lo cual puede ser explicado como una expansión de la población post-glacial del refugio de los Balcanes.

4.1.2. Variación en las frecuencias de los ordenamientos cromosómicos

Los estudios de los cambios en las frecuencias del polimorfismo de inversión después de la invasión de *D. subobscura* en el Nuevo Mundo demuestran que las clinas latitudinales se desarrollaron aproximadamente dentro de los 5 primeros años, sugiriendo una evolución rápida y predecible después de la colonización (Balanyà *et al*., 2003), y señalando igualmente que dichos cambios son una respuesta a los gradientes ambientales, principalmente a la temperatura. Sin embargo, los resultados en conjunto de este estudio muestran una falta de correspondencia entre los resultados de selección termal de laboratorio y las predicciones basadas en las clinas latitudinales establecidas después de la colonización del Nuevo Mundo, dado que únicamente los efectos de la temperatura fueron significativos para los ordenamientos $A_{st,}$ E_{st} y $O_{3+4+2.}$

El patrón de variación del ordenamiento A_{st} fue congruente con las clinas latitudinales observadas, (aumento de la frecuencia de los ordenamientos estándar a latitudes más altas y frías, Menozzi y Krimbas 1992; Orengo y Prevosti 1996), pero no así el ordenamiento E_{st} que, aunque mostró un índice de cambio muy rápido similar al de las poblaciones naturales, lo hizo en sentido opuesto (aumenta la frecuencia cuando se incrementa la temperatura). Por otro lado, el ordenamiento O_{3+4+2} es el único caso de los ordenamientos cromosómicos con inversión en el que coinciden los resultados de laboratorio y las tendencias naturales (aumento de la frecuencia de los ordenamientos con inversión a las latitudes más bajas y cálidas, Menozzi y Krimbas 1992).

Por tanto, se puede decir que los patrones naturales de la variación clinal del polimorfismo cromosómico no parecen en general ser causados por un efecto directo de la temperatura, y que posiblemente otros factores climáticos asociados con la latitud pueden estar influyendo en la formación de dichos patrones en las poblaciones colonizadas del Nuevo Mundo.

4.1.3. Evolución termal de las características del ala

Aproximadamente 20 años después de presentarse las clinas geográficas para el polimorfismo cromosómico en Suramérica y Norteamérica, se registraron también clinas latitudinales para la longitud del ala que fueron morfológicamente equivalentes a las clinas originales del Viejo Mundo. Esta situación, junto a los experimentos de evolución termal, en los que la adaptación a bajas temperaturas resulta consistentemente en un incremento del tamaño corporal (Anderson 1966; Powell 1974; Cavicchi *et al.*, 1989; Patridge *et al.,* 1994), han llevado a la mayoría de las investigaciones a concluir que las clinas geográficas en tamaño del cuerpo en *Drosophila* son el resultado de la adaptación a diferentes temperaturas.

4.1.3.1.Tamaño del ala

Los resultados de este trabajo muestran que, aunque hay un efecto del régimen de selección termal que es evidente dos años después de establecidas las poblaciones experimentales, con una disminución de la longitud promedio del tamaño del ala (WL) y el tamaño del centroide (CS) a la temperatura optima (18ºC), no hay indicación de una tendencia lineal de cambio con la temperatura. Además las poblaciones adaptadas al calor (moscas criadas a 22ºC) no fueron más pequeñas que sus equivalentes adaptadas al frío (moscas criadas a 13ºC) y por el contrario sus longitudes promedio fueron similares en ambas temperaturas extremas (13ºC y 22ºC), por lo que no es completamente cierto que la adaptación a baja temperatura conlleva invariablemente a la evolución de un tamaño corporal más grande en *Drosophila*.

Estos resultados, contrarios a las conclusiones de los estudios previos de evolución termal, podrían tener explicación en primer lugar debido al poco tiempo transcurrido de evolución termal en el laboratorio, dado que los estudios similares que han demostrado cambios en el tamaño corporal en poblaciones experimentales, las han mantenido por largos periodos de tiempo (Anderson 1973; Cavicchi *et al.,* 1989; Patridge *et al.,* 1994a), y en segundo lugar, por la falta de control en las condiciones de cría de los estudios anteriores, cuyos resultados podrían haber sido dependientes del régimen de crecimiento larval. Todo lo cual confirmaría lo propuesto por Partridge y French (1996), de que el crecimiento larval parece tener un papel importante en el establecimiento de las diferencias en tamaño del cuerpo en experimentos de selección termal y, por tanto, podría tener también un papel en el establecimiento de las clinas del tamaño corporal.

4.1.3.1.1. El tamaño celular y el número celular

Por otro lado, los análisis de los componentes celulares, el área celular y el número celular, muestran un patrón similar al descrito para la longitud promedio del tamaño del ala, con una interacción significativa entre temperatura y sexo, aunque con una respuesta más representativa en hembras a nivel de área celular (que disminuye a 18ºC), mientras que en machos es más importante el número celular (que disminuye igualmente a 18ºC). Esto permite, en términos generales, decir que la reducción mencionada para el tamaño del ala en el régimen de selección óptimo (18ºC), parece estar mediada tanto por el área celular como por el número celular, lo cual parece confirmar lo encontrado por Partridge *et al.* (1999), donde la respuesta correlacionada con el tamaño decreciente del ala depende tanto del área celular, como por del número celular, contrariamente a lo propuesto por otros, donde las diferencias en el tamaño del ala son completamente una consecuencia de la divergencia del tamaño celular (Robertson 1959; Cavicchi *et al.,* 1985; Partridge *et al.,* 1994a). De esta manera, se puede decir que los detalles por los que las clinas de tamaño se desarrollaron parecen ser contingentes con un origen en la base celular de las diferentes clinas de tamaño (Calboli *et al.,* 2003a), o en el segmento en particular del ala causante de la clina (Huey *et al.,* 2000; Santos *et al.,* 2004).

4.1.3.1.2. Relación entre el polimorfismo de inversión y la variación clinal del tamaño del ala

Las diferencias en tiempo de aparición entre las clinas latitudinales para polimorfismo cromosómico (5 años después de la colonización) y para tamaño del ala (20 años después) (Huey *et al.,* 2000; Gilchrist *et al.,* 2001, 2004; Calboli *et al.,* 2003a), sugieren que el tamaño corporal en las poblaciones del Nuevo Mundo probablemente no está relacionado

con los ordenamientos génicos implicados en las clinas latitudinales. Asimismo, los resultados de este estudio muestran claramente que las clinas latitudinales para el polimorfismo de inversión cromosómico y el tamaño corporal están desacopladas, no hay una relación positiva entre los ordenamientos génicos y la longitud promedio del ala, aunque si un efecto significativo de los cromosomas A, E y O en el tamaño corporal. Lo que resulta particularmente importante ya que estos cromosomas son homólogos a los que han sido implicados en el control de las clinas latitudinales del tamaño corporal en *D. melanogaster* (2R y 3R, Gockel *et al.,* 2002; Calboli *et al.,* 2003b) y en *D. buzzatti* (cromosoma 2 homólogo al cromosoma O de *D. subobscura*).

Las correlaciones entre el tamaño del cuerpo y las inversiones polimórficas han sido observadas en diferentes especies de *Drosophila* (Betrán *et al.,* 1998; Stalker 1980; Van Delden y Kamping 1991; Gockel *et al.,* 2001, 2002; Calboli *et al.,* 2003b; Knibb *et al.,* 1981; Knibb 1982; Weeks *et al.,* 2002; James *et al.,* 1995; Van't Land *et al.,* 2000) y particularmente en muestras europeas de *D. suboscura* que apuntan a una relación positiva entre el tamaño del ala y la dosis del ordenamiento génico estándar en esta especie (Prevosti 1967; Orengo y Prevosti 2002; Krimbas 1967). Sin embargo, los resultados tan diversos en estos estudios, muestran simplemente la dificultad a la hora de entender los efectos del polimorfismo de inversión de *Drosophila* en la variación clinal del tamaño corporal.

En lo que respecta al presente estudio, los resultados no excluyen lo observado para las clinas de tamaño corporal del Viejo Mundo (Prevosti 1955; Misra y Reeve 1964; Pfriem 1983; Pergueroles *et al.,* 1995) y por el contrario, se corresponden con la falta de correlación en las muestras analizadas del Nuevo Mundo, lo cual podría explicarse como un producto del efecto fundador que ocurrió durante la colonización.

4.1.3.2. Forma del ala

En contraste con las clinas latitudinales claramente definidas para el tamaño del ala, no existe un patrón consistente entre la latitud y la forma del ala en *Drosophila*. En *D. subobscura* el índice de forma (L1/WL) aumenta con la latitud en Europa y disminuye en Norteamérica (Huey *et al*., 2000), pero no muestra ninguna tendencia lineal en Suramérica (Gilchrist, comunicación personal 2002).

En lo que respecta a este estudio, el índice de forma, así como los análisis de variación de forma del ala, no revelan ninguna tendencia lineal con la temperatura, pero exhiben un efecto

significativo del régimen de selección termal. El índice de forma muestra que la porción basal del ala (L1) en hembras incrementa a las temperaturas extremas (13°C y 22°C) en contraste con la temperatura óptima (18°C), y en machos es significativamente más alta únicamente a la temperatura de 13°C, lo que es compatible con el patrón de contraste hallado en las poblaciones de la región paleártica (una correlación positiva entre el índice de forma y la latitud) y en las poblaciones recién colonizadas de Norteamérica (una correlación negativa con la latitud; Huey *et al.,* 2000). El efecto significativo del régimen de selección termal en la variación de forma del ala en ambos sexos, está asociado a cambios localizados en determinados *landmarks*, con algunas ligeras diferencias entre sexos. Los efectos de la variación entre individuos están distribuidos en forma relativamente igual entre todos los *landmarks* y con fuerte dependencia entre ellos. Estos exhiben un movimiento en la dirección de un aspecto ampliado y estrecho del ala, así como una relación inversa entre la longitud del segmento basal o proximal (aumento L1) y la longitud del segmento distal (disminuye L2) de la vena longitudinal IV, tal y como se ha observado ya en los estudios de Gilchrist *et al*., (2001) en muestras europeas y norteamericanas.

En general, los contrastes entre los patrones para forma y tamaño del ala de este estudio, así como en otros estudios en la misma línea (Weber 1990, 1992, Weber *et al*., 1999, 2001; Bitner-Mathe y Klaczko 1999 a y b; Birdsall *et al*., 2000; Klingenberg y Zaklan 2000; Zimmerman *et al*., 2000; Gilchrist y Patridge 2001), sólo parecen indicar que hay diferentes propiedades genéticas paradel la forma y el tamaño ala, las cuales muestran una variación profusamente estructurada y localizada para la forma.

4.1.3.2.1. Tasas de divergencia genética para forma del ala

La tasa de evolución del tamaño del ala y su divergencia, estimadas a escala continental, son consideradas muy rápidas en *D. subobscura* (Huey *et al*., 2000; Gilchrist *et al*., 2001). Sin embargo, la estima de las tasas de divergencia evolutiva del cambio de forma del ala en las poblaciones experimentales, demuestran que dichas tasas pueden ser tan rápidas o incluso más rápidas que aquellas estimadas para el tamaño del ala (Gilchrist *et al*., 2001). Hay una variación de forma significativa y consistente a través de las poblaciones replicadas para el régimen de selección termal en ambos sexos, con diferencias significativas detectadas por comparación "post hoc" cuando se contrastaron las temperaturas de 18ºC vs 13ºC o 22ºC en hembras, y 13ºC vs 22ºC en machos.

Esta alta tasa de divergencia genética en un periodo corto de evolución termal en laboratorio, podría explicarse en relación al carácter adaptativo de los cambios de forma del ala. La forma del ala responde a selección divergente en el mismo sentido que la mayoría de los caracteres cuantitativos, con algunos genes causando pequeños efectos localizados (Weber *et al*., 1990, 1992). Esto sugiere abundantes cambios en el desequilibrio de ligamiento de las inversiones en las especies de *Drosophila subobscura* ricas en inversiones, particularmente en muestras derivadas de poblaciones fundadoras colonizadoras del Nuevo Mundo.

4.1.3.2.2. Relación entre el polimorfismo de inversión y el índice de forma del ala

El stock de selección termal ha divergido para varios ordenamientos génicos sin diferencias entre las poblaciones replicadas. Sin embargo, se observa una relación fuerte entre las formas variables y las tendencias de la dosis estándar; concretamente, el índice de forma disminuye marcadamente cuando se incrementa la dosis de los ordenamientos cromosómicos estándar, lo que parece dejar claro un papel del polimorfismo de inversión de *D. subobscura* en el efecto biométrico en la forma del ala y la importancia de aquellos en la formación de las clinas de forma en las poblaciones colonizadas de Norteamérica (Huey *et al*., 2000). Por lo tanto, los cambios en las frecuencias de los ordenamientos génicos como una respuesta a la temperatura, probablemente subrayan los cambios correlacionados en la forma del ala debido al desequilibrio de ligamiento presente en las inversiones génicas.

Por otro lado, este estudio revela indicios posibles de que las clinas de forma en Norteamérica sean anteriores a las clinas de tamaño, dadas las diferencias en tiempo marcadas entre el desarrollo de las clinas del polimorfismo de inversión y las de tamaño, así como, por la posibles relaciones entre los ordenamientos cromosómicos estándar y las clinas de forma del ala. Los análisis realizados con los datos reportados por Pergueroles *et al*., (1995) cuatro años después de la colonización inicial en Norteamérica, y antes de que la clina de tamaño se desarrollara, parecen ser concluyentes, ya que confirman que el índice de forma disminuye con la latitud. Esta tendencia es igualmente evidente en muchas muestras recientes (Gilchrist *et al*., 2001), lo que pone en duda el supuesto de “incertidumbre o imprevisibilidad” de las clinas geográficas de *D. subobscura* en las poblaciones colonizadas norteamericanas (Huey *et al*., 2000).

4.2. Análisis de los efectos de la variación genética en las dimensiones del ala, en líneas isocromosómicas del cromosoma O

Los organismos suelen ser resistentes a las alteraciones tanto genéticas como ambientales y suelen presentar un fenotipo normal. La producción de estos fenotipos consistentes u óptimos se halla muy condicionada a los mecanismos de control de canalización y estabilidad del desarrollo, implicados en el amortiguamiento que mantienen la variación inter e intra individual baja, durante las perturbaciones que aparecen en la morfogénesis. En este sentido, la forma del ala en *Drosophila* se ha considerado fuertemente resistente a las influencias ambientales (Weber 1990; Birdsall *et al*., 2000), dadas las evidencias de que la evolución del desarrollo y la temperatura, relacionadas con la divergencia del tamaño celular tienen efectos contrastantes en la forma del ala. Sin embargo, en *D. subobscura* se han observado cambios en las porciones del ala a lo largo de una clina latitudinal de tamaño mediados por el área celular (Huey *et al*., 2000; Calboli *et al*., 2003a), así como también se ha observado en la primera parte de este trabajo, que estos cambios latitudinales en las porciones del ala están bajo la misma línea que clinas latitudinales para el polimorfismo de inversión (tendencias de la dosis estándar).

Dentro de este marco, es importante saber hasta que punto la variable de forma del ala (tamaño y forma) en *D subobscura* presentan una fuerza genética y fenotípica contra las perturbaciones ambientales, que explique la plasticidad aparente en la respuesta de estos caracteres a los factores tanto ambientales de la naturaleza, como a los de los diferentes estudios de selección termal de laboratorio. Para ello, en esta segunda parte del estudio se ha realizado un análisis genético cuantitativo que permite ver los posibles efectos de la variación genética clinal (polimorfismo de inversión) en las dimensiones tamaño y forma, y en la simetría bilateral del ala, usando líneas isocromosómicas del cromosoma O ($O_{st,}$ $O_{3+4\ y}$ O_{3+4+7}) de *D. subobscura*, teniendo en cuenta las consecuencias de la endogamia y la temperatura en dos de los componentes de la homeostasis del desarrollo, la canalización y la estabilidad del desarrollo (DS), y la relación entre ambos.

4.2.1. Variación y asimetría en el tamaño del ala

Los análisis de tamaño muestran en general que no hay efectos de antisimetría y alometría, como tampoco diferencias significativas de tamaño entre los cariotipos, pero sí interacciones del efecto de dominancia en las muestras con heterosis perceptible en las líneas O_{st}/O_{3+4} comparadas con sus contrapartes homocariotípicas.

La DS esta positivamente correlacionada con los niveles de heterocigosis (endogámicas vs homocariotípicas exogámicas) y desarrollo a la temperatura optima de 18°C, y se observan efectos de consanguinidad (depresión endogámica) en las líneas endogámicas principalmente a 23ºC, pero no se detectan interacciones de la temperatura por cariotipo, lo que demuestra que el tamaño del ala no es una característica puramente aditiva en *D. subobscura*, según las observaciones previas en cruces exogámicos, en las que los heterocariotipos fueron más grandes que los homocariotipos (Pfriem 1983).

Asimismo, los efectos de endogamia y temperatura son también evidentes en hembras a 23ºC, con un alta FA, aunque sin ninguna diferencia aparente entre cariotipos. En machos la endogamia parece afectar la variación entre moscas a las temperaturas de cría (18ºC y 23ºC) en los cruces exogámicos. Es importante mencionar también, que en los cruces exogámicos la FA del tamaño total, fue la misma tanto en homocariotipos como en los heterocariotipos, y el único efecto significativo fue nuevamente un incremento en la FA a la temperatura de 23ºC. Por lo que se puede decir que no hay ninguna asociación positiva entre la DS y la heterocigosis cromosomal en cruces exogámicos.

4.2.2. Variación y asimetría en la forma del ala

Se detectada una variación genética, principalmente para la forma total del ala, en la componente direccional de la asimetría morfológica (DA), que bien podría indicar la presencia de variación genética a nivel de un eje del desarrollo izquierdo – derecho filogenéticamente conservado, como es discutido por Klingenberg *et al.* (1998).

Los análisis de los componentes genéticos de la FA en la forma del ala en cruces exogámicos, no detectaron ninguna variación genética en ninguna de las dos temperaturas experimentales, como tampoco ninguna variación genética detectable para la FA de la forma del ala, cuando se usó la medida escalar de la cantidad de asimetría de forma en la serie de datos. Lo que probablemente refleja variación debida a efectos estocásticos en el desarrollo.

Asimismo, ciertas diferencias entre sexos son evidentes en algunas situaciones. Concretamente, se observa efectos endogámicos de consanguinidad en la FA de la forma del ala únicamente en hembras a la temperatura de 18ºC, así como en la FA del tamaño total a la temperatura de 23ºC principalmente en hembras. Hay un efecto considerable de la temperatura y la endogamia en el componente no alométrico en la forma del ala para ambos sexos, pero un efecto alométrico en la forma del ala únicamente en machos. Estas diferencias sexuales ya se

evidenciaban en las clinas de tamaño del ala que se desarrollaron en *D. subobscura* 20 años después de la colonización, en la que las hembras demostraron clinas mucho mas definidas en contraste con la de los machos (Gilchrist *et al*., 2001).

4.2.2.1. Patrones de la variación de la forma del ala

En los análisis de forma, los patrones de variación para FA y el error de medida fueron altamente concordantes en todas las muestras, sugiriendo que la FA es generada por perturbaciones al azar por procesos del desarrollo, aunque esto no implica que la DS es independiente del fondo genético, ya que la FA de la forma del ala fue mas alta en hembras endogámicas criadas a 18ºC cuando se comparo con sus homologas no endogámicas (exogámicas homocariotípicas) criadas a la misma temperatura.

En los análisis de forma, las comparaciones totales por correlación de matrices de los cariotipos asociadas con los niveles de variación de los componentes genéticos interindividual (canalización) o intraindividual (FA como DS) en general, mostraron una baja correlación (cercana a cero o incluso negativa). Un resultado que se apoya también, con la baja similaridad encontrada en la comparación de los vectores correspondientes que describen los patrones de la variación de la posición de los *landmarks*. Por tanto, esta falta de similaridad o significación fue básicamente debida a las discrepancias de los componentes genéticos interindividuales de canalización y los componentes ambientales de la variación interindividual, que sugieren que la canalización genética y ambiental no son mecanismos similares, y por tanto la canalización ambiental y DS comparten mecanismos reguladores subyacentes, pero no son idénticos.

Por otro lado, considerando los 6 cariotipos exogámicos heterocariotípicos se encontró el mismo patrón de acortamiento de la longitud basal de la vena longitudinal IV en la longitud total del ala respecto a la dosis estándar, como ya se observó en la primera parte de este trabajo. Y aunque la temperatura afectó el índice de forma, no hubo ningún cariotipo significativo en interacción con la temperatura. Además, los heterocariotípos siempre fueron intermedios a sus contrapartes correspondientes homocariotípicas, por lo que el índice de forma del ala parece ser una característica puramente aditiva, con pequeños efectos aditivos en la forma por parte de muchos genes que están dispersos a lo largo del genoma (Weber *et al.,* 1999, 2001; Zimmerman *et al.,* 2000).

Sin embargo, aunque las clinas de forma del ala en las poblaciones colonizadas de América de *D. subobscura* (Huey *et al.,* 2000) pueden ser en gran parte paralelas a las clinas latitudinales

de los ordenamientos cromosómicos, y el pequeño cambio del cruce de la vena anterior y posterior puede tener relación con la variación cariotípica, es difícil relacionarlo con alguna respuesta adaptativa. Los resultados del trabajo por tanto sólo hacen aportes a la conjetura de Gilchrist *et al.,* (2001) de una variación de la forma del ala representando simplemente el flujo alrededor de un óptimo, ya que la canalización genética en la forma del ala no parece surgir como un producto de la canalización ambiental, por lo que, la canalización no es un mecanismo sencillo para amortiguar alguna fuente de variación como ha sido sugerido (Meiklejohn *et al.,* 2002).

Los patrones de variación debidos a los componentes ambientales de canalización (efectos especiales de covarianzas ambientales entre moscas) fueron completamente similares a estos observados para el FA. La varianza ambiental entre los individuos genéticamente idénticos, o canalización (micro) ambiental, dada por la FA de los dos lados que comparte el mismo conjunto de genes y que además, actúan en las mismas condiciones ambientales, podría ser pequeña (limite inferior teórico de canalización). Bajo la selección estabilizadora este límite muy bajo es obviamente asociado con una mayor aptitud. Sin embargo, este "limite de canalización" podría no ser visto a causa de factores adicionales que pueden verse implicados en la variación de los valores de FA como por ejemplo, la variación entre tubos, la posición de la pupa en un tubo, etc.

Finalmente, considerando los resultados en conjunto sobre la congruencia entre patrones de variación morfológica con respecto a la variación atribuible a FA y la variación genética atribuida por DA en los cruces exogámicos criados a 18ºC, se pone en duda si la FA y variación genética para DA están funcionalmente ligadas.

5. CONCLUSIONES

Los resultados de la evolución termal de laboratorio del polimorfismo de inversión, y las dimensiones del ala en las poblaciones experimentales de *Drosophila subobscura* concluyen que:

- El polimorfismo de inversión ha respondido rápida y consistentemente a la temperatura, pero los cambios observados en las frecuencias de muchas ordenaciones cromosómicas no son consistentes con las predicciones basadas en las clinas latitudinales o con las tendencias observadas en las poblaciones naturales de América y Europa.
- La evolución térmica observada para el carácter tamaño del ala tampoco es consistente con la observación común de que individuos derivados de latitudes superiores suelen ser más grandes que los derivados de latitudes inferiores, por lo que no es completamente cierto que la adaptación a baja temperatura conlleva necesariamente a la evolución de un tamaño corporal más grande en *Drosophila*.
- Los análisis de la forma del ala revelan diferencias consistentes y significativas con la temperatura pero sin ninguna tendencia lineal, y por el contrario sus cambios parecen estar relacionados con la dosis de los ordenamientos estándar, que revelan la importancia que pueden tener estos en la formación de las clinas de forma del ala en las poblaciones de Norteamérica.
- Por lo tanto, ninguna de las clinas latitudinales (polimorfismo de inversión, tamaño y forma) parece tener un origen por efecto de la temperatura, o al menos no es el único factor responsable de la formación de las clinas latitudinales.

Los resultados del análisis de los efectos de la variación genética en las dimensiones del ala, en líneas isocromosómicas del cromosoma O, indican que;

- La canalización ambiental y la estabilidad de desarrollo comparten mecanismos reguladores subyacentes, pero la canalización ambiental y genética no son funcionalmente lo mismo, posiblemente debido a que la variación de forma del ala en las poblaciones naturales de *Drosophila* está indirectamente relacionada con la aptitud individual.

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