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RESEARCH ARTICLE

Enhanced fertility and chill tolerance after cold-induced reproductive arrest in females of temperate species of the *Drosophila buzzatii* complex

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ABSTRACT

Long-term exposure to low temperatures during adult maturation might decrease fertility after cold recovery as a consequence of carry-over effects on reproductive tissues. This pattern should be more pronounced in tropical than in temperate species as protective mechanisms against chilling injuries are expected to be more effective in the latter. We initially determined the lower thermal thresholds to induce ovarian maturation in four closely related *Drosophila* species, two inhabiting temperate regions and the other two tropical areas of South America. As expected, only temperate species regularly experience cold-inducing conditions for reproductive arrest during winter in their natural environment. Subsequently, we exposed reproductively arrested and mature females to cold-inducing conditions for reproductive arrest over a long period. Following cold exposure, tropical species exhibited a dramatic fertility decline, irrespective of reproductive status. In contrast, not only were temperate females fecund and fertile but also fertility was superior in females that underwent cold-induced reproductive arrest, suggesting that it might act as a protecting mechanism ensuring fertility after cold recovery. Based on these findings, we decided to evaluate the extent to which reproductive status affects cold tolerance and energy metabolism at low temperature. We found a lower metabolic rate and a higher cold tolerance in reproductively arrested females, although only temperate species attained high levels of chill tolerance. These findings highlight the role of cold-induced reproductive arrest as part of an integrated mechanism of cold adaptation that could potentially contribute to the spread of temperate species into higher latitudes or altitudes.

KEY WORDS: Cold resistance, Insect, Metabolic rate, Ovarian arrest, Triglycerides, Overwintering

INTRODUCTION

Selective pressures vary considerably across seasons. As a consequence, reproductive performance can be strongly influenced by dynamic environmental challenges, resulting in the optimization of different components of fitness during the year. At higher latitudes, for example, fitness of ectotherms relies on the ability to exploit the warm season for growth, development and reproduction, as well as the ability to mitigate the negative effects of winter cold by lowering metabolism and arresting reproduction (Bradshaw and Holzapfel, 2010). Reproductive recovery with mild effects on fertility as a result of long-term cold exposure is expected by the end of the winter season, in response to more benign conditions.

The genus *Drosophila* consists of three thousand species (Markow and O'Grady, 2005) and represents a vast collection of organisms adapted to a wide variety of environmental challenges. Some species have adapted to severely cold climates characteristic of high latitudes and altitudes. For instance, cold tolerance is strongly increased by reproductive arrest in *Drosophila suzukii* (Toxopeus et al., 2016). Another cold-adapted species is *Drosophila montana* (*virilis* group); flies of this species can tolerate temperatures below 0°C for approximately half of the year, undergoing reproductive diapause, a hormonally mediated arrest in response to a token environmental stimulus (Vesala and Hoikkala, 2011). *Drosophila melanogaster*, in contrast, represents a different situation. Although this species originated in a tropical area, its status as a genetic model organism permitted important insights into the metabolic and genomic mechanisms involved in the interplay between cold tolerance and reproductive arrest (Kubrak et al., 2014). Although this has not been well tested, cold-adapted species are expected to maintain fertility after prolonged exposure to cold in order to succeed at high latitudes (Mockett and Matsumoto, 2014; Marshall and Sinclair, 2010).

The *Drosophila repleta* species group, to which the species studied here belong, is a monophyletic group of Neotropical flies that has diversified in the Western Hemisphere, adopting a cactophilic lifestyle that allows them to thrive in the American deserts (Wasserman, 1982). Some of the *repleta* species belong to the *mulleri* species complex and are endemic to the Sonoran Desert in Mexico and southern Arizona, where they confront extreme heat and desiccation stress. Likewise, South America witnessed the radiation of the *buzzatii* complex (sister group to the *mulleri* complex). The *buzzatii* complex is composed of three clusters of closely related species that inhabit South American deserts: the *martensis* cluster in northern South America, the *stalkeri* cluster in the Caribbean area and the *buzzatii* cluster in the southern cone of South America. This vast distribution range implies that some of these species face a wide variety of thermal regimes. Taking into consideration this historical biogeographic scenario, a tropical lifestyle is likely to be the ancestral state of South American cactophilic flies. On this evolutionary scale, moving into higher latitudes and altitudes imposed strong selective pressures as a result of cooler winter seasons, promoting the evolution of adaptive

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overwintering mechanisms. In this study, we focus on four South American cactophilic species of the *buzzatii* complex (Fig. 1A). *Drosophila borborema* (*buzzatii* cluster) and *Drosophila venezolana* (*martensis* cluster) inhabit tropical deserts (Fig. 1B) where environmental temperatures are high and mostly uniform throughout the year (Fig. 1C), indicating that these species do not need to cope with low temperature challenges. In contrast, *Drosophila buzzatii* and *Drosophila koepferae* (*buzzatii* cluster) reach higher latitudes and altitudes in subtropical and temperate regions (Fig. 1B) where winters are very cold (Fig. 1C), suggesting that these species can tolerate low temperatures and have the capacity to recover reproductive activity when optimal environmental conditions are restored.

In the present study, we exploited the ecological features of these four species and their evolutionary relationships (Fig. 1) to address the effect of long-term cold exposure on energy metabolism, cold tolerance and fitness. Our first goal was to assess reproductive recovery in temperate and tropical species after long-term cold-induced reproductive arrest. To address this issue, groups of females of temperate species were exposed to long-term low temperatures and distinct photoperiod conditions, after which their ovaries were examined to evaluate the degree of sexual maturity. Our prediction was that females would show immature ovaries, indicating that cold-induced reproductive arrest is indeed possible. Furthermore, following a shift to higher temperatures, we expected females to recover mating receptivity after several days and to be fecund and fertile; in other words, they would not suffer fitness reduction as a consequence of exposure to prolonged cold. We expected that females of species living in tropical deserts would also have immature ovaries after cold treatment but, conversely, would be unable to recover normal mating acceptance and fertility when transferred to more benign conditions. This expected reproductive impairment in tropical flies would be due to the lack of mechanisms to protect reproductive tissues against chilling injuries.

Adapting to temperate conditions can also require a large number of changes in dealing with cold tolerance as well as shifting metabolic optima. If temperate and tropical flies differ in their capacity to recover fertility after long-term cold exposure, they would also provide an opportunity to investigate the interplay of reproductive arrest and other aspects of overwintering physiology such as cold tolerance and energy metabolism at low temperature. In this context, we predicted that reproductive arrest would only increase cold tolerance in temperate species (not in tropical species), as they are naturally exposed to low-temperature conditions. Finally, we examined nutrient and energy metabolism during cold exposure. Although reproductive arrest confers a metabolic depression by which nutrient reserves are conserved, metabolic pools are substantially depleted during long-term cold exposure (Hahn and Denlinger, 2011). We therefore expected that such metabolic depression would only be observed in temperate species, as they evolved the capacity to enter reproductive arrest. Thus, we predicted that temperate species would show decreased consumption of metabolic reserves relative to tropical species during long-term exposure to low temperature.

MATERIALS AND METHODS

A summary of the experimental procedure is given in Fig. 2. In short, newly emerged females of tropical and temperate species were exposed to two thermal regimes. The first consisted of a steady cold acclimation treatment and the second consisted of a thermal shift treatment, in which an initial warm temperature was followed by cold acclimation. This experimental design allowed us to compare females of the same age with contrasting reproductive

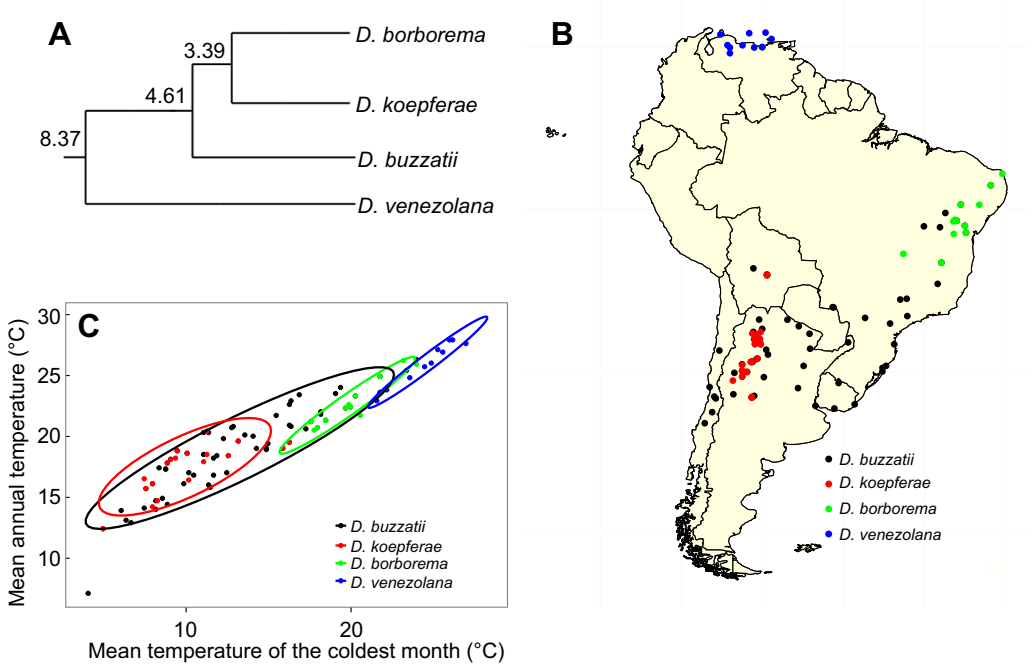

Fig. 1. Phylogenetic relationship, distribution and environmental temperature during the coldest month for *Drosophila* species belonging to the *buzzatii* complex. (A) Phylogeny of South American *Drosophila* species belonging to the *buzzatii* complex. Divergence times among species range from 8.37 to 3.39 Mya, with *D. venezolana* being the most diverged of the group (Oliveira et al., 2012). (B) Geographical distribution of species in South America. Dots represent geographical coordinates of localities of the different populations of temperate (*D. buzzatii* and *D. koepferae*) and tropical (*D. borborema* and *D. venezolana*) species. (C) Winter and annual temperatures for South American cactophilic *Drosophila* species. Mean temperature of the coldest month and mean annual temperature are depicted for each geographical coordinate. Ellipses enclose 90% of the data for each species.

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Journal of Experimental Biology (2017) 220, 713-721 doi:10.1242/jeb.150540

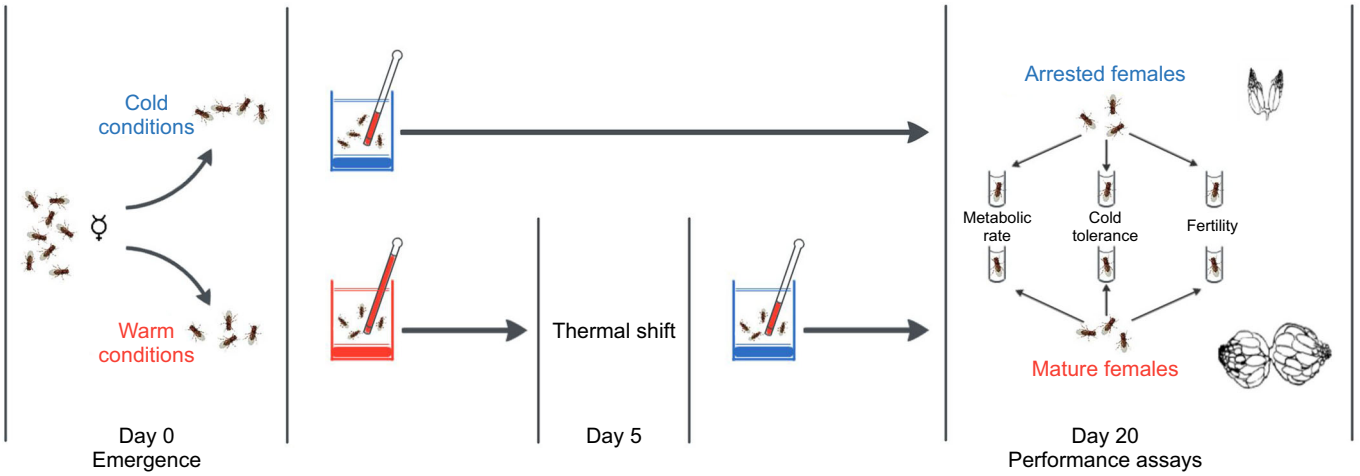

Fig. 2. Summary of the experimental procedure employed in the study. Newly emerged females of tropical and temperate species were exposed to two thermal regimes: the first consisted of a steady cold acclimation treatment and the second consisted of a thermal shift treatment, in which an initial warm temperature was followed by cold acclimation. The initial warm condition allows ovarian maturation. Subsequently, we compared the responses of the two groups (arrested and mature females) and species in terms of energy metabolism, cold tolerance and fertility.

status (arrested versus mature), conditioned by a common low temperature acclimation. Subsequently, we compared the responses of both groups and species in terms of energy metabolism, cold tolerance and fertility (Fig. 2). Details of the experimental groups are given in 'Energy metabolism, cold tolerance and fertility in temperate and tropical species', below.

South American *Drosophila* species studied

In this study, we included one isofemale line of each of four species from contrasting biogeographic origins: two temperate species, *D. buzzatii* and *D. koepferae*, and two tropical species, *D. borborema* and *D. venezolana*. The *D. buzzatii* line derived from the progeny of a female collected in Vipos, Argentina (26°32′ S, 65°14′W), and the *D. koepferae* line from Ruinas de Quilmes, Argentina (26°35′S, 65°55′W). The *D. borborema* line was originally sampled in Morro do Chapeau, Brazil (11°42′S, 41°28′ W), and obtained from UC San Diego *Drosophila* Stock Center and the *D. venezolana* line was sampled in Isla de Los Roques, Venezuela (11°46′N, 66°44′W), and kindly donated by Antonio Fontdevila (Universidad Autónoma de Barcelona, Spain) (see Cerda and Fontdevila, 1998, for details).

Quantifying the lower thermal limit to induce ovarian maturation

Experimental lines were reared at 25°C and under a 12 h:12 h light: dark regime at low density on an instant mashed potato medium hydrated with a water solution of the antifungal Nipagin (*p*-hydroxybenzoic acid methyl ester) (Sassi and Hasson, 2013). Groups of 200 females <6 h post-emergence were exposed to five different cold treatments (10, 12, 14, 16 and 18°C) for 20 days, combined with two alternative photoperiods simulating long (14 h:10 h light:dark) and short (10 h:14 h light:dark) days, making a total of 10 treatments. After 20 days of cold exposure, all females were dissected to evaluate the degree of ovarian development in each of the 10 conditions. Based on ovarian condition, we defined two types of females: reproductively arrested (RA) and mature females. RA females were defined by the presence of only pre-vitellogenic ovarioles in both ovaries (stages 1–7 according to King, 1970), while mature females had at least one stage 8 (vitellogenic) oocyte in either ovary.

Eco-geographical analysis to assess the incidence of reproductive arrest across species in nature

We obtained the geographical coordinates of all localities where the presence of a particular species has been reported (http://www.taxodros.uzh.ch/ and from personal communications of various collectors). Subsequently, for each locality, we obtained the temperature of the coldest month from WorldClim (www.worldclim.org) and then utilized these data to verify whether winter temperatures at those sites are below the critical temperature that effectively produces the arrest of ovarian development in each species.

Energy metabolism, cold tolerance and fertility in temperate and tropical species

Two different groups of females of each species were exposed to distinct thermal treatments (Fig. 2). A steady cold acclimation group consisted of a cohort of immature females, sexed within 6 h of emergence from the puparium and maintained for 20 days at 10°C for temperate and 12°C for tropical species. These temperatures are the upper thermal limits that elicit arrest of ovarian maturation in temperate and tropical species, respectively (see Results, 'Lower thermal limit for ovarian maturation'). The thermal shift treatment group was established with females that were maintained at 25°C for 5 days post-emergence to allow full ovarian maturation. Subsequently, mature females were transferred to chambers at either 10 or 12°C, for temperate and tropical species, respectively, for the next 15 days. This experimental design allowed us to compare females of the same age in contrasting reproductive status (arrested versus mature), conditioned at a common low acclimation temperature. The thermal shift treatment group thus served as a maturation reference for the subsequent metabolic, cold tolerance and fertility assays.

Metabolic pool quantification

We compared metabolic reserves between RA females and newly emerged females. For these measurements, sets of five cold-acclimated or newly emerged females of each species were used in the quantification of glycogen and triglycerides (five replicates were used for each combination of treatment group and species). Each set of five females was dried at 50°C for 5 days. Fly dry mass was

Journal of Experimental Biology (2017) 220, 713-721 doi:10.1242/jeb.150540

measured to the nearest 0.1 mg using an analytical balance (Mettler AJ100, Toledo, OH, USA), then flies were homogenized in 1 ml of phosphate buffer (25 mmol l^{-1} KH_2PO_4, pH 7.4) and centrifuged for 3.5 min at 14,300 ***g***. A total of 850 µl of the supernatant was removed and frozen. Glycogen content was measured using the PGO Enzymes kit (Wiener Lab, Rosario, Argentina) with the addition of 0.1 U of amyloglucosidase (Sigma-Aldrich, St Louis, MO, USA) per ml of reaction buffer. Samples (50 µl of homogenate +1 ml of reaction buffer) were incubated at 25°C for 30 min and absorbance was determined in a microplate reader (Biotek ELx808, Bad Friedrichshall, Germany) at 490 nm. Triglyceride content in each sample was determined by adding 1 ml of the Triglyceride Reagent Set (Wiener Lab) to 10 µl of homogenate. The resulting solution was incubated at 37°C for 10 min prior to absorbance quantification at 505 nm in a microplate reader (Biotek ELx808). As this study focuses on energy storage compounds, total lipid content (which includes structural lipids) was not determined, and only triglycerides were measured. Metabolic pools were measured in triplicate per sample, standardized by fly dry mass, and mean values were used for further analysis.

Metabolic rate measurements

We measured real-time CO_2 emission as a proxy for metabolic rate in six (replicates) groups of 20 flies of each reproductive status (RA and mature) by species combination, using a Sable Systems International (SSI; www.sablesys.com) flow-through respirometry device. Females were placed in the absence of cold or CO_2 anesthesia inside the respirometric chamber. Recordings lasted approximately 40 min. Trial temperatures matched the thermal treatments of each species, and were controlled by a SSI Pelt-5 temperature controller coupled to a SSI Peltier Plate. Chamber temperature was measured by a thermocouple attached to a SSI TC-2000 thermocouple meter. H_2O- and CO_2-free air was drawn at a flow rate of ca. 50 ml min^{-1} by a SS4 sub-sampler (SSI) through low-permeability, Bev-A-Line tubing and entered a SSI RC-M precision thermal respirometry chamber. In addition, the activity of the insects was simultaneously monitored and recorded by a SSI AD-2 activity detector (see Lighton and Schilman, 2007; Schilman et al., 2011, for details). Air leaving the chamber carrying CO_2 produced by the flies entered a Li-Cor CO_2 infrared analyzer (LI-6251, Lincoln, NE, USA; resolution 0.1 ppm CO_2). Baseline recordings were taken at the beginning and end of each trial with an empty chamber. Finally, the flies were chilled and weighed to the nearest 0.1 mg using an analytical balance (Mettler AJ100).

The analog outputs from the CO_2 analyzer, fly activity detector, thermocouple meter and air flow rate meter were connected to an A/D converter (SSI UI-2, 16-bit, basic accuracy=0.05%) and stored on a computer using ExpeData data acquisition and analysis software (SSI). Data were sampled at 1 Hz. Several corrections and conversions were made from the recordings (see Rolandi et al., 2014, for details).

Mass-independent metabolic rate was calculated by dividing $\dot{V}_{CO_2}$ (µl h^{-1}) by live mass (mg) raised to the 0.856 power, which is the inter-specific mass scaling exponent for tracheate arthropods (Lighton et al., 2001). Given that the trial temperature varied between measurements of tropical and temperate species, metabolic rates were compared assuming a Q_{10} of 2.

To compare activity from recordings of different lengths, an index of locomotion activity was calculated. To this end, activity was converted to the absolute difference sum (ADS), i.e. the cumulative sum of the absolute difference between all adjacent data points, and the index of activity was calculated using Eqn 1:

$$\text{Activity index} = \text{ADS range}/N \times 60, \quad (1)$$

where ADS range is the difference between maximum and minimum values of ADS activity and N is the number of seconds or samples of the recording.

Only partial regions of the recordings where the activity index was lower than 16 ADS range/minutes were utilized in order to discard differences in metabolic rate mainly due to activity.

Cold-tolerance assay

Following 20 days of thermal treatment, 50 females of both groups (RA and mature) of each species were transferred without anesthesia to vials set in boxes containing water at 0°C. After 12 h at 0°C, females were allowed to recover at 25°C and recovery from chill coma was individually measured as the time (in minutes) elapsed until flies could stand up on their legs (David et al., 1998).

Fertility measurements

Another set of RA and mature females was transferred to room temperature (25°C) and individually crossed with a pair of mature males. Once mating had taken place, batches of five females (for each reproductive status by species combination) were allowed to lay eggs for 7 days in vials on lab medium. On day 7, females were discarded and the number of adult progeny was recorded daily from the emergence of the first fly up to 10 days. RA and mature females of the same species were tested in parallel. In addition, a control group (not cold acclimated) was set up with 5-day-old virgin females which passed through the crossing scheme as described above but were never subjected to cold (always maintained at 25°C).

Statistical analyses

All statistical analyses were computed in R version 3.1.1. For each variable analyzed, we first used a complete model with all possible predictor terms and interactions. We then removed non-significant terms that failed to increase the model goodness of fit. We evaluated this by applying the *anova* function of the *stats* package (http://www.R-project.org/), which tests for reduction in the residual sum of squares.

For the analysis of ovarian arrest under different environmental conditions, a logistic regression model was applied to the entire dataset using the *glmer* function of the *lme4* R package (http://CRAN.R-project.org/package=lme4). The model included environmental temperature as a continuous predictor, biogeographic origin (tropical or temperate) as a fixed factor, species (four levels) nested in biogeographic origin as a random factor, and the biogeographic origin × environmental temperature and species × environmental temperature interactions. Photoperiod and the interactions involving this factor were excluded from the model as none of these terms proved to significantly affect ovarian development or increase the model goodness of fit (χ^2_2=1.557, P=0.4591).

Table 1. Logistic regression analysis of environmental factors affecting reproductive arrest in *Drosophila* species of different biogeographic origin (temperate/tropical)

Effect	Estimate	*P*-value
Temperature	1.17	<0.001
Biogeographic origin	−13.38	<0.05
Temperature×biogeographic origin	0.67	0.13

Journal of Experimental Biology (2017) 220, 713-721 doi:10.1242/jeb.150540

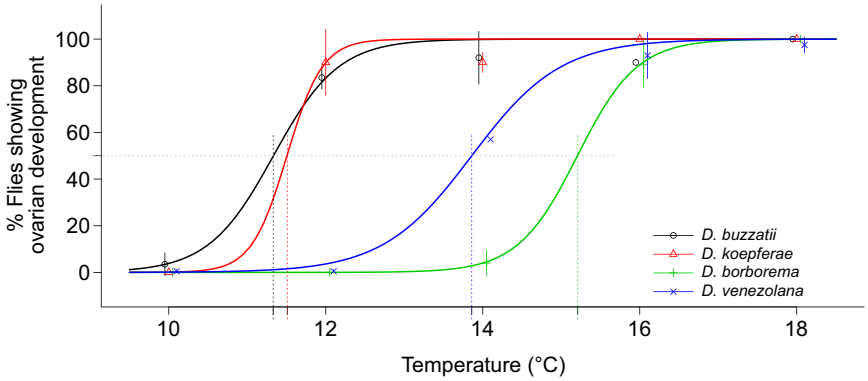

Fig. 3. Lower thermal limit for ovarian maturation for temperate (*D. buzzatii* and *D. koepferae*) and tropical (*D. borborema* and *D. venezolana*) *Drosophila* species. Ovarian development was scored as the presence of at least one stage 8 (vitellogenic) oocyte in either ovary. Vertical dashed lines indicate the thermal threshold for the induction of ovarian development. Tropical species showed 50% induction of ovarian development at 13.9°C (*D. venezolana*) and 15.2°C (*D. borborema*), whereas temperate species did not reach 50% ovarian arrest until the temperature fell to 11.3°C (*D. buzzatii*) and 11.5°C (*D. koepferae*).

To examine how flies consume metabolic reserves during cold acclimation, a residual maximum likelihood (REML) approach was applied for fitting a mixed effects model (*lmer* function of the *lmerTest* packages). The model included the biogeographic origin and metabolite (glycogen and triglyceride) as fixed factors, species as a random factor nested in biogeographic origin and the biogeographic origin × metabolite interaction. The species × metabolite interaction was excluded from the model as it failed to increase the model goodness of fit.

To test the effects of biogeographic origin and reproductive status on metabolic rate, cold tolerance and fertility, a REML approach was applied for fitting a mixed effects model (*lmer* function of the *lmerTest* packages, see http://CRAN.R-project.org/package=lmerTest). Each model included the following terms: biogeographic origin and reproductive status (RA and mature) as fixed factors, species as a random factor nested in biogeographic origin and the biogeographic origin × reproductive status interaction. For metabolic rate and fertility analyses, the interaction species × reproductive status failed to increase the model goodness of fit and was excluded from both models. Chill coma recovery time data were log transformed.

RESULTS

Lower thermal limit for ovarian maturation

Our first hypothesis predicted that low temperature would differentially affect the arrest of ovarian development in tropical and temperate species. In effect, the temperate species (*D. buzzatii* and *D. koepferae*) attained ovarian maturity at moderately low temperature, whereas the tropical species (*D. borborema* and *D. venezolana*) required an environmental temperature of about 3–4°C higher to reach maturity (Table 1, Fig. 3). Specifically, ovarian maturation as a function of temperature was fitted to a logistic curve (Fig. 3), and from its equation, we could infer that 50% of *D. buzzatii* and *D. koepferae* females attained ovarian maturation at critical temperatures of 11.3 and 11.5°C, respectively, while 50% of *D. venezolana* and *D. borborema* females attained maturity at 13.9 and 15.2°C, respectively.

Subsequently, we analyzed the records of mean temperature during the coldest month in localities of all populations of tropical and temperate species in order to assess the occurrence of proper field conditions that may lead to arrest of reproduction by low temperature. Climatic data for the different biogeographic regions where the species are currently found indicate that tropical and temperate species are exposed to distinctly different conditions in nature (Fig. 1C). The largest fraction of *D. koepferae* and *D. buzzatii* populations experience conditions that prevent female maturation during the coldest month of the year at least. In contrast, *D. venezolana* and *D. borborema* populations never experience such cold conditions during the warm tropical winters in their geographical range, confirming that only temperate species are likely to experience cold-induced reproductive arrest in nature. Based on these findings, we decided to test the predictions stemming from the biogeographical patterns by evaluating the extent to which cold-induced reproductive arrest differentially affects fitness-related aspects in tropical and temperate species.

Metabolic reserves

We compared glycogen and triglyceride content of cold-induced RA females with newly emerged females for all species. After 20 days of cold exposure, glycogen content significantly decreased in comparison to triglyceride reserves (P=4.99×10^{-07}; Table S1). Glycogen consumption was consistent across species (Fig. 4), with an overall decrease of 49.8% relative to newly emerged females. Additionally, while RA females of temperate species gained 16% body mass in comparison with newly emerged flies, tropical species experienced a body mass reduction of about 10% (although the

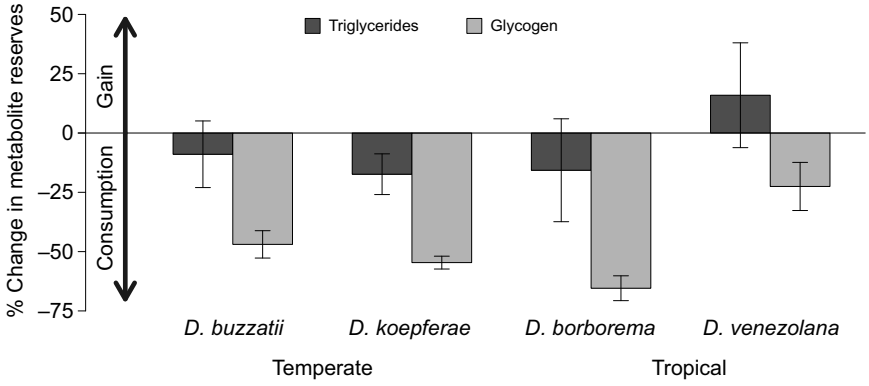

Fig. 4. Percentage change in metabolic reserves for temperate and tropical *Drosophila* species after 20 days of cold acclimation. Data (means±s.e.m.) for the metabolite (triglycerides and glycogen) content are expressed relative to the content of newly emerged females of the different species.

Journal of Experimental Biology (2017) 220, 713-721 doi:10.1242/jeb.150540

Table 2. Residual maximum likelihood analysis of metabolic rate, chill coma recovery time and fertility in reproductively arrested and mature females of *Drosophila* species with different biogeographic origins (temperate/tropical)

	Metabolic rate		Chill coma recovery		Fertility	
Effect	Estimate	*P*-value	Estimate	*P*-value	Estimate	*P*-value
Biogeographic origin	0.005	0.95	34.45	0.18	–35.56	<0.001
Reproductive status	0.147	<0.001	7.31	<0.05	–15.33	<0.001
Biogeographic origin×reproductive status	–0.116	<0.05	23.12	<0.001	16.5	<0.001

difference between tropical and temperate species was not statistically significant).

Metabolic rate

Metabolic rate can vary as a consequence of locomotion activity, and although parts of the recording with low and similar activity were selected for the analyses, this factor cannot be ignored. Thus, we added this factor into the model and, as expected, metabolic rate varied with activity (estimate=0.009, P=0.04). Nonetheless, reproductive status and its interaction with biogeographic origin were highly significant (Table 2, Fig. 5).

To further explore this interaction, we analyzed how metabolic rate varied between reproductive states of each species. As reproductive arrest implies a reduction in energy metabolism, we expected metabolic depression to only affect females of temperate species, as they are naturally exposed to cold-inducing conditions during winter (see above). Contrary to our expectation, not only temperate species but also *D. borborema* showed a metabolic rate decline in RA females (Fig. 5). Such similarity across species may indicate a common mechanism of metabolic depression in response to reproductive status.

Cold tolerance

We predicted that acclimation at low temperature would enhance cold tolerance. Specifically, our expectation was that reproductive arrest would increase cold tolerance in temperate species as only these species are naturally exposed to severe cold winters. Our results show that chill coma recovery time was affected by the interplay between reproductive status and species biogeographic origin, as indicated by the significant origin × reproductive status interaction (Table 2). Although RA flies recovered faster than mature flies in all species, only temperate species attained a high level of cold tolerance after cold acclimation (Fig. 6). The fastest chill coma recovery time (1.5 min at room 25°C) was observed in *D. buzzatii* females following cold-induced reproductive arrest. After 12 h at 0°C, fly mortality was negligible except for mature *D. venezolana* females, which exhibited a 39% decrease in survival.

Fertility

If reproductive arrest is adaptive, the fertility of temperate species should remain unaltered compared with that of females not exposed to cold. However, cold acclimation should impose detrimental effects on the reproductive success of females from tropical species. As expected, we detected strikingly different reproductive output among species after cold acclimation. In temperate species, RA females maintained high reproductive output following cold recovery similar to that of non-acclimated flies (Table 3). In contrast, in tropical species, the number of adult progeny fell dramatically after long-term exposure to cold irrespective of reproductive status (Table 3). In addition, RA females exhibited a fertility advantage compared with mature females only in temperate species (Fig. 7). In effect, the number of adult offspring was much higher in RA females compared with mature females in *D. koepferae* ($F_{1,18}$=15.47, P=0.0009), whereas differences were marginally significant in *D. buzzatii* ($F_{1,40}$=3.87, P=0.056). Finally, given that temperate females recovered mating behavior within a few days of cold recovery (Table S2), our results suggest that they underwent reproductive quiescence.

DISCUSSION

An important assumption made in many studies investigating the ecology, physiology and evolution of overwintering in insects is that after cold acclimation insects maintain high fertility. However, reproductive recovery after long-term exposure to low temperatures is rarely tested. In this study, we determined the lower thermal limit to induce ovarian maturation in four closely related *Drosophila* species, two inhabiting temperate regions and the other two tropical areas of South America. Our comparative approach revealed that fertility following cold acclimation below this thermal limit showed the most striking differences between the temperate and tropical species studied in this paper. The capacity for delayed reproduction along with the improvement of cold tolerance suggest the adaptive significance of reproductive arrest in the life cycle of temperate *Drosophila* species such as *D. buzzatii* and *D. koepferae*. Moreover, as tropical lifestyle is likely

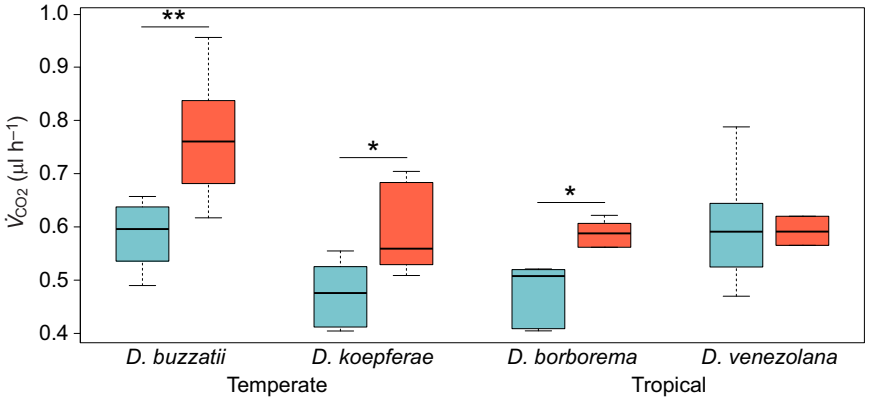

Fig. 5. Metabolic rate ($\dot{V}_{CO_2}$) of reproductively arrested (blue) and mature (orange) *Drosophila* females of temperate and tropical species. Six replicates per group were run. *P<0.05 and **P<0.01 using ANOVA to compare reproductive status within species. Boxplot lines represent the median and box boundaries are the upper and lower quartiles (e.g. 25th percentiles). Error bars represent the maximum and minimum values.

Journal of Experimental Biology (2017) 220, 713-721 doi:10.1242/jeb.150540

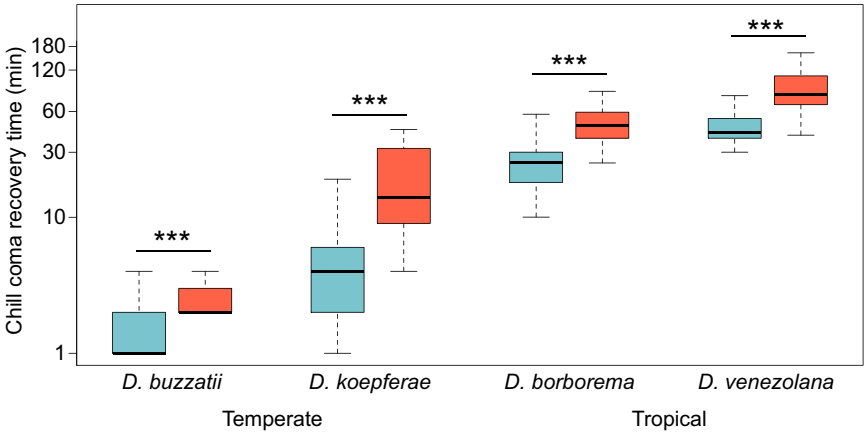

Fig. 6. Chill coma recovery time of reproductively arrested (blue) and mature (orange) *Drosophila* females of temperate and tropical species. Rapid chill coma recovery depends on the interplay between reproductive status and biogeographic origin. Sample sizes for each species (reproductive arrest, mature): *D. buzzatii* (*N*=33, *N*=37), *D. koepferae* (*N*=26, *N*=33), *D. borborema* (*N*=25, *N*=26) and *D. venezolana* (*N*=26, *N*=29). ***P<0.001 using ANOVA to compare reproductive status within species. Boxplot lines represent the median and box boundaries are the upper and lower quartiles (e.g. 25th percentiles). Error bars represent the maximum and minimum values.

the ancestral state in the group of South American cactophilic flies studied, the present results indicate that the ability to enter reproductive arrest upon the arrival of cold conditions would have allowed them to colonize cooler environments, i.e. higher latitudes or altitudes. Further studies, however, involving a larger number of tropical and temperate species are required in order to establish the generality of our results.

Incidence of reproductive arrest in temperate and tropical *Drosophila*

Following cold acclimation, temperate females not only were fecund and fertile but also their fertility was higher in RA compared with mature females. Cold-induced reproductive arrest might act as a protective mechanism ensuring fertility following cold recovery. In contrast, tropical species exhibited a dramatic fertility decline after cold exposure, irrespective of reproductive status. As observed in other cold-acclimated insects, several agents may be involved in the protection of reproductive tissues against chill injury in temperate *Drosophila* species. For example, numerous heat shock proteins (Hsps) (Rinehart et al., 2007; Colinet et al., 2010) and antioxidants (Sim and Denlinger, 2011) play an important role in protection during cold stress. Further investigations should assess the involvement of such mechanisms in females of temperate *Drosophila* species under cold-induced reproductive arrest. Another possible explanation for the reduction of fertility in mature females relative to RA females may be that the latter were physiologically younger than mature females despite both groups being of the same chronological age.

Our evidence indicates that in the southern-most populations of *D. buzzatii* and in a large fraction of the *D. koepferae* distribution range, the mean temperature during the coldest month of the year is below the lower thermal limit to induce ovarian development (Fig. 1), meaning that along a wide range of temperate distributions, the environmental conditions are suitable to induce reproductive arrest during the winter.

Effects of reproductive arrest on cold tolerance and energy metabolism

We hypothesized that adapting to temperate conditions would require a large number of changes in dealing with cold tolerance as well as shifts in metabolic optima. In contrast to our expectations, we found common aspects of energy metabolism across species irrespective of their biogeographic origin. For instance, both temperate and tropical species rely primarily on carbohydrate metabolism during reproductive arrest, as observed in other cold-acclimated drosophilids (Marshall and Sinclair, 2010). In addition, cold-induced RA females of all species down-regulated metabolic rate in comparison with mature females, except for *D. venezolana* (Fig. 5). Given that, in nature, tropical species face conditions that are above the thermal threshold that elicits cold reproductive arrest, the sharing of the reduction in metabolic rate across species may be a relictual plastic response that was present in the common ancestor of the *buzzatii* complex, despite the likely tropical origin of these flies. Alternatively, the reduction in metabolic rate may have been shaped by other stressors, such as desiccation, common to all cactophilic flies (Gibbs et al., 2003; Matzkin and Markow, 2009). A prediction of the latter interpretation is that similar responses are expected in other desert-adapted species. Given that global warming is occurring faster in winter than in summer (IPCC, 2007), an increase of thermal variability during reproductive arrest may generate negative effects in terms of fitness by increasing the consumption of stored energy reserves (Williams et al., 2012). This ecological trap (Van Dyck et al., 2015) would also raise the risk of mortality during the cold season.

Finally, our study demonstrates that cold-induced reproductive arrest improves chill tolerance in temperate species. Although RA flies recovered faster than mature flies in all species, only temperate species attained high levels of cold tolerance after cold acclimation (Fig. 6). Remarkably, RA *D. buzzatii* females recovered within less than 2 min, showing the highest tolerance to cold exposure. Similarly, the Andean species *Drosophila pavani* exhibits great cold tolerance after acclimation at 10°C (Boher et al., 2010),

Table 3. Comparison of fertility between non-acclimated and cold-acclimated [reproductively arrested (RA) and mature] females

	Non-acclimated	RA	Mature	ANOVA
D. buzzatii	46.4±6.99 (5)	37.81±6.89 (10)	28.38±2.18 (11)	$F_{1,23}$=2.40; P=0.1130
D. koepferae	40.0±4.0 (5)	44.06±5.54 (8)	22.39±2.61 (12)*	$F_{1,22}$=9.62; P=0.0009
D. borborema	13.5±0.87 (8)	5.77±1.08 (8)**	7.32±1.22 (18)**	$F_{1,31}$=7.79; P=0.0018
D. venezolana	51.55±2.58 (6)	4.16±0.63 (7)***	3.94±0.74 (9)***	$F_{1,19}$=365.20; P<0.0001

Values correspond to the mean (±s.e.m.) number of emerged adults per female (the number of replicates is given in parentheses). Non-acclimated flies were used as a control within each species. *P<0.05, **P<0.01 and ***P<0.001 using ANOVA followed by Dunnett tests to compare non-acclimated and cold-acclimated females in each species.

Journal of Experimental Biology (2017) 220, 713-721 doi:10.1242/jeb.150540

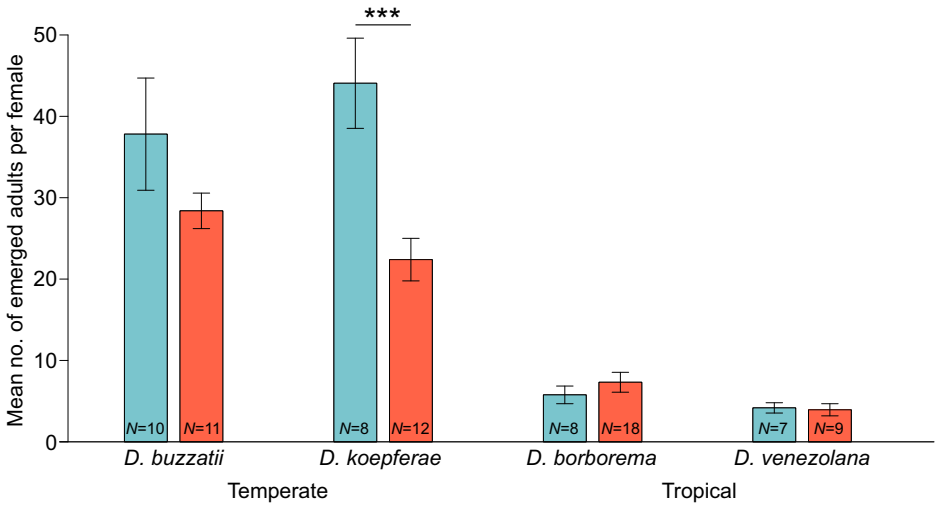

Fig. 7. Number of adult offspring of reproductively arrested (blue) and mature (orange) females of temperate and tropical species. Data are the mean (±s.e.m.) number of adult progeny derived from a batch of five females. The number of adult offspring was recorded daily from the emergence of the first fly up to 10 days. The number of replicates for each species by treatment is shown in the bars. ***P<0.001 using ANOVA to compare arrested and mature females within species.

suggesting that this species may also undergo cold-induced reproductive arrest.

In addition to the acclimatizing plastic responses of adults to different environmental conditions, the diverse thermal environments experienced by animals during ontogeny may also shape thermal tolerance (Bowler and Terblanche, 2008). In the light of enhanced cold tolerance and down-regulation of energy metabolism in RA females, we hypothesized that South American temperate species of the *buzzatii* complex could undergo reproductive diapause, a hormonally mediated arrest of reproduction in response to a token stimulus (Koštál, 2006). Further investigations should assess the effect of cold acclimation on early developmental stages and utilize additional species in order to find out the role of an endocrine signal modulating an anticipatory arrest of reproduction during winter.

In conclusion, our findings stress the need to take into account reproductive arrest as a cold-tolerance mechanism and its consequences on modeling current and future distributions of species that inhabit temperate zones of the globe.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

This study was conceived and designed by J.M., J.H., P.E.S. and E.H. Experiments were carried out by J.M., J.H., P.F.Z. and C.R. J.M., J.H., G.d.l.V. and C.R. analysed the data. J.M., T.A.M. and E.H. wrote the first draft, and all authors contributed to and approved the final version.

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Supplementary information

Supplementary information available online at
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