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PII: S0306-4565(23)00045-1

DOI: https://doi.org/10.1016/j.jtherbio.2023.103504

Reference: TB 103504

To appear in: *Journal of Thermal Biology*

Received Date: 20 October 2022

Revised Date: 2 February 2023

Accepted Date: 3 February 2023

Please cite this article as: Kreiman, L., Putero, F., Hasson, E., Mensch, Juliá., Extended lifespan and sex-specific fertility loss in cold-acclimated flies of the sibling species *Drosophila buzzatii* and *Drosophila koepferae*, *Journal of Thermal Biology* (2023), doi: https://doi.org/10.1016/j.jtherbio.2023.103504.

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Methodology: LEK, FP

Formal analysis: LEK

Original draft: LEK, JM & Review: LEK, EH, FP, JM

Supervision: EH; JM

Project administration: JM

Extended lifespan and sex-specific fertility loss in cold-acclimated flies of the sibling species *Drosophila buzzatii* and *Drosophila koepferae*

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Abstract

Survival and reproduction are the core elements of Darwinian fitness. In the context of a fixed energy budget, organisms tend to allocate resources in order to maximize one at the expense of the other, in what has been called the lifespan-reproduction trade-off. Reproductive arrest and extended lifespan are common responses to low temperatures in many insects including fruit flies. In this study, we aim to understand the overwintering strategy of two closely-related *Drosophila* species with contrasting distribution ranges. We compared survival, lifespan, ovarian maturation, and reproductive output (fecundity and fertility) of virgin and mated adults of both *Drosophila buzzatii* and *Drosophila koepferae* after long-term cold exposure at dormancy-inducing conditions (10°C, 10:14 L:D) and controls (25°C, 12:12 L:D). Virgin flies of *D. buzzatii* showed the longest lifespan (averaging 102 days) under dormancy-inducing conditions. Cold-induced reproductive arrest preserves reproductive capacity mainly in virgin females that mated after reproductive dormancy, indicating that males were much more susceptible to fertility loss than females, in both species. Notably, females of *D. buzzatii* were capable of protecting stored sperm from cold damage and produced viable progeny. Even if, in *D. buzzatii*, fertility of flies mated after the cold-exposure was extremely low, cold temperature likely sterilized *D. koepferae* males, indicating that cold carry-over effects are stronger for the species with the shorter lifespan. Such species-specific effects of low temperature over fitness likely contributed to the divergence of these closely-related species and to the spread of *D. buzzatii* into cooler environments.

Keywords: Reproductive dormancy; Cold adaptation; Fruit fly; Overwintering.

1. Introduction

To mitigate the effects of low temperatures during winter, many adult arthropods adopt a variety of dormant responses usually associated with reduced metabolism and increased stress torelance. When such a physiological state is an anticipatory response and is triggered by a short photoperiod it results in a case of diapause (Bradshaw & Holzapfel, 2007; Kostal, 2006). Diapause is a deep interruption and redirection of the developing program that evolved in several orders of insects (Beck, 1980). Instead, another form of dormancy is quiescence, which consists of a direct response to low temperature on metabolism (Kostal, 2006, Saunders, 1982). Diapause and quiescence can occur at various developmental stages in different species. In the case of temperate *Drosophila* species, overwintering adults usually enter a state of reproductive dormancy, which is associated with the arrest of mating behavior in both sexes (Tatar & Yin, 2001, Ala-Honkola, et al, 2018), detention of accessory gland activity in males (Tatar & Yin, 2001), and arresting ovarian maturation in females (Toxopeus et al., 2016, Mensch et al., 2017, Lirakis et al., 2018; Panel et al, 2020, Ala-Honkola, et al, 2018, Lavagnino et al., 2020). The overwintering stage of most temperate *Drosophila* species is the adult stage (reviewed in Lumme & Lakovaara, 1983), as adults are commonly more cold-tolerant than pre-adult stages (Izquierdo, 1991; Enriquez & Colinet, 2017) and winter acclimatization can boost adult cold tolerance. For example, in temperate regions, overwintering survival of *D. melanogaster* and *D. suzukii* populations mainly rely on adults, as juvenile stages are more susceptible to cold temperatures (Izquierdo, 1991; Stockton et al., 2018; Enriquez & Colinet, 2017). By the end of the winter season, though carry-over effects of low temperature might cause damage in reproductive tissues, populations resume growing. Current evidence in *Drosophila*

suggests that males are more sensitive to fertility loss than females (Panel et al., 2020), however, the generality of this pattern is still in debate (Iossa, 2019; Walsh et al, 2022). Sex-specific thermal fertility limits can result in complex overwintering strategies. If males suffer reduced fertility, population persistence and build-up could rely on overwintering females carrying fertile sperm. Alternatively, virgin females and fertility-reduced males could reproduce once the winter season is over.

In the context of a fixed energy budget, dormant organisms tend to maximize survival at the expense of reproduction, in what has been called the Y-Model or the lifespan-reproduction trade-off (de Jong & van Noordwijk, 1992; Reznick, 1985; Tatar & Carey, 1995; Stearns, 1989). Notably, *D. melanogaster* post-dormant flies after resuming reproduction showed a similar age-dependent mortality rate as non-dormant flies, indicating a slowed senescence during reproductive dormancy (Tatar et al., 2001). Post-dormancy reproduction, in contrast, declined with the duration of the cold treatment; implying that somatic survival during dormancy may tradeoff with late reproduction. In effect, in many *Drosophila* species, reproduction at low temperature usually has a detrimental effect on longevity, as mated flies have a shorter lifespan than their virgin counterparts in both sexes (Boulétreau-Merle & Foulliet, 2002; Panel et al., 2020). Thus, it is expected that virgin rather than mated flies have a higher chance of successfully overwintering.

The *Drosophila repleta* species group, to which the species studied here belong, is a monophyletic group of Neotropical flies (Oliveira et al., 2012) that has diversified in the Western Hemisphere, adopting a cactophilic lifestyle that allows them to thrive in the American deserts (Wasserman, 1982, Hasson et al. 2019). In this study, we focus on two South American cactophilic sibling species, *Drosophila koepferae* and *Drosophila buzzatii,* that diverged about 1.54 million years ago (Hurtado et al., 2019,

Moreyra et al., 2019, Moreyra et al., 2022). Both species inhabit temperate and subtropical regions of the south-central area of South America. Moreover, these species are the only members of the *buzzatii* cluster that reach locations 2000 meters above sea level, indicating that they can tolerate low temperatures. With some overlap, *D. buzzatii* and *D. koepferae* have different ranges of distribution, as *D. buzzatii* extends wider in the latitudinal axis of South America (Mensch et al., 2017), suggesting that *D. koepferae* could be more sensitive to extreme thermal conditions. There are few studies that directly measure thermal tolerance of both species simultaneously (Scannapieco et al., 2007; Mensch et al., 2017, Mensch et al., 2021). A survey of heat tolerance has shown that *D. koepferae* is more resistant than *D. buzzatii* only in young (4-day-old) flies, but not when tested at older age (11-day-old), suggesting a faster rate of senescence in *D. koepferae* than in *D. buzzatii* (Scannapieco et al., 2007). Regarding cold tolerance, females of both species showed similar chill-coma recovery time and Critical Thermal Minimum after dormancy-inducing conditions (Mensch et al., 2017, Mensch et al., 2021). Moreover, females of both species maintained high reproductive output after dormancy-inducing conditions (Mensch et al, 2017). In contrast, males of *D. buzzatii* exhibited a fertility loss as a consequence of cold developmental acclimation (Vollmer et al., 2004). Thus, it is expected that males of *D. koepferae* show a similar fertility loss after cold-exposure, but this question has not been addressed so far. Importantly, it has been observed that *D. koepferae* showed a higher early fecundity but a much faster senescence rate than *D. buzzatii* under benign conditions (25ºC), results that are coherent with the trade-off between fecundity and longevity (Fanara et al., 1999; Sambucetti et al., 2005; Soto et al., 2012). Considering that thermal sensitivity can vary even between closely-related species (Behrman et al. 2015), it would be worth

comparing the effect of dormancy-inducing conditions on longevity and fertility in both species and sexes, simultaneously, testing for differences in their fundamental thermal niches linked to overwintering.

The present study aimed to investigate how dormancy-inducing conditions affect lifespan and reproduction and search for possible overwintering strategies in the pair *D. buzzatii* and *D. koepferae.* To this end, we measured several fitness-related traits (e.g. lifespan, survival, ovarian maturation, fecundity, and fertility) in flies of both species maintained under dormancy-inducing conditions for several weeks and the corresponding controls. In addition, we explored whether pre-cold mated females are capable of protecting stored sperm from cold damage and produce viable progeny after cold exposure. If that is the case, we predict, however, a survival cost of reproduction, *i.e.*, mated flies would show a reduced lifespan than their virgin counterparts. Finally, we expect a stronger carry-over effect of cold-acclimation on male fertility.

2. Materials and Methods

2.1 Fly collections and stock maintenance

We studied three isofemale lines (lines from hereafter) of *D. buzzatti* and three of *D. koepferae* derived from collections in San Agustín del Valle Fértil, Argentina (31°7′4.08″ S, 67°40′42.96″ W). Prior to the experiments, lines were maintained for over a year (about 20 generations) under regulated humidity (70%), photoperiod (12:12), and temperature (25°C) and fed as described in Mensch et al. (2017) with instant mashed potato medium hydrated with a water solution of the antifungal Nipagin (p-hydroxybenzoic acid methyl ester) and yeast as a source for protein.

2.2 Experimental design

A summary of the experimental setup showing details of the experimental conditions and traits studied is shown in Figure 1. The specific protocols of each trait are described in the following sections.

2.2.1 Survival and longevity

We aimed to assess the effect of mating status (virgin vs. mated) on adult survival and longevity under dormancy-inducing conditions. To this end, we measured mortality of mated and virgin individuals of both sexes and species exposed to 10°C and under a 10L:14D photoperiod, simulating winter season conditions at the collecting site (http://siga.inta.gob.ar/; Table S1). Previous studies have shown that such low temperature combined with winter photoperiod induce reproductive arrest in both *D. buzzatii* and *D. koepferae* (Mensch et al., 2017). To obtain virgins flies, individuals were collected daily upon emergence and immediately separated by sex and exposed to dormancy-inducing conditions. For the group of mated flies we applied the following protocol: newly emerged flies were sorted by sex and kept at 25°C for 7 days until they reach sexual maturation, and then were crossed in dyadic encounters. After copulation, flies were sorted by sex and placed in vials under dormancy-inducing conditions. Vials were checked for fly survival every 2-3 days and survivors were transferred to new vials with fresh medium every week until the death of the last fly. The same culture medium described above was employed in all treatments. Simultaneously, we run a survival assay at 25°C, and under a 12L:12D photoperiod, for both virgin and mated flies and both sexes and species as controls. We run a factorial design including the following factors: mating status (mated vs. virgin), acclimation conditions (dormancy-inducing conditions vs. control), species

(*D. buzzatii* and *D. koepferae*), and sexes, with an average of 13 replicates per combination. Each replicate consisted of 10 individuals of either sex.

2.2.2 Ovarian maturation

We evaluated sexual maturity in the group of virgin females by analyzing the state of ovarian development along the survival assay under dormancy-inducing conditions. To do so, we inspected the ovaries of naturally died females that were periodically removed from the vials of the Survival assay (see section 2.2.1 and fig. 1A). Simultaneously, random samples of an average of 28 alive- females were removed every 15 days and sacrificed via exposure at -4°C to score ovarian maturation. In these assays nine time-points were evaluated over the course of 135 days. A total of 1420 female flies were dissected, 914 females of the group of dead females, and 506 females of the group of alive ones. A total of 456 vials (replicates) were analyzed, 288 were employed for periodically sampling alive-females and 168 corresponded to the group of dead females. Ovaries were dissected and studied under a microscope at 40x magnification. An ovary was considered mature when it showed at least one oocyte at Stage 8 (vitellogenic) according to King's classification (King, 1970).

2.2.3 Reproductive output

The aim of this experiment was to evaluate and compare the carry-over effect of long-term cold-exposure on reproductive capabilities (*i.e.*, fecundity and fertility) of "pre-cold mated flies", "post-cold mated females", "post-cold mated males" and "post-cold mated flies". We also included a control group of mated flies not exposed to cold. The group of "pre-cold mated flies" consisted of virgin 7 day-old mature flies

196 of both sexes kept at 25°C that were crossed in dyadic encounters before the cold
197 treatment at 10°C for six weeks (Fig. 1 B). In turn, post-cold crossings were
198 generated using virgin flies of both sexes that were transferred, after six weeks at
199 10°C, to 25°C for seven days to allow sexual maturation. Then, to obtain post-cold
200 mated females, single cold-exposed females were mated with one 7-day old virgin
201 male kept at 25°C. To generate post-cold mated males, single cold-exposed males
202 were mated with one 7-day old virgin female kept at 25°C. Finally, the group of post-
203 cold mated flies consisted of the crossings of cold-exposed females and cold-
204 exposed males after maintained at 10°C for six weeks. We chose a six-week period
205 of cold-acclimation as survival analyses showed that survival functions remained
206 unaltered during that lapse of time across experimental groups (see section 3.1). By
207 doing this, we assessed the reproductive output in a period unaffected by survival.
208 Crossings occurred between 9:00 and 11:00 am (i.e. the peak of daily sexual
209 activity) in small, translucent vials. For each mated female, the number of eggs laid
210 during the first 7 days (early fecundity) was counted daily, as well as the hatching
211 rate (fertility). Each fly was individually placed in a device described in Figure S1 to
212 count the number of laid eggs. A 1% agar gel was prepared and poured into small
213 Petri dishes. Once agar solidified, a small amount of dry yeast was added over the
214 surface as stimulus for oviposition and food for both adults and, eventually, larvae.
215 Dishes were replaced once a day and observed under the microscope for egg
216 counting. Two days later, we counted the number of hatched larvae as a measure of
217 fertility.
218
219 2.3 Statistical Analyses

To evaluate the differential responses among experimental groups, Generalized Lineal Mixed Models (GLMMs) were performed using the “glmmTMB” (Brooks et al., 2017) package for R (R Core Team, 2022). This package allows dealing with heteroskedasticity when needed. For the analyses of ovarian maturation, fecundity and fertility, the models included species and reproductive status as fixed factors, and line as random effect nested within species. For the analyses of survival and longevity, the models included mating status, instead of reproductive status, and also temperature and sex as fixed effects. Models included the interaction between fixed factors. The “DHARMa" package was used to test for normality and heteroscedasticity (Hartig, 2021). We performed Analyses of Deviance (type II Wald Chi-squared likelihood ratio tests), which are the GLMs equivalent of an analysis of variance, using the ‘Anova()' function of the “car” package (Fox & Weisberg, 2019). In case of significant interactions, differences among groups were tested by means of *post-hoc* Tukey’s tests using the “emmeans” package (Lenth et al., 2021). We employed the “ggplot2” package (Wikcham, 2016) for data visualization.

2.3.1 Survival and longevity

For the survival analysis, we utilized two complementary approaches: a Cox’s mixed model (COXME) and a Generalized Additive Mixed Model (GAMM) (Bender et al., 2018). The former builds a survival function that represents the probability of individual death at a given time assuming continuous time (Cox, 1972). In this case, we used “survival” (Therneau & Grambsch, 2000) and “coxme” (Therneau, 2018) packages, which allow random effects. In contrast, a Generalized Additive Mixed Model (GAMM) builds a survival function by adding up several functions through intervals of discrete time. The “mgcv” package (Wood, 2017) was used to generate the GAMM analyses, while “pammtools” package (Bender & Scheipl, 2018) allows a

clear representation of survival functions. The optimal number of basis functions was determined by the 'gam.check()' function of the "mgcv" package.

We run a specific survival analysis for the flies under dormancy-inducing conditions (10°C, 10:14 L:D). For this, temperature was excluded as a variable. We did model selection using Akaike Information Criterion (AIC), resulting in the models that included sex as an additive factor.

Longevity was measured as age at death (in days) and fitted to GLMM with a normal distribution using the "glmmTMB" package, as previously described.

2.3.2 Ovarian maturation

Ovarian maturation was measured as the proportion of mature females within each vial and the model accounted for the effect of age (in days), status (whether flies were alive or dead when taken from the incubator), species, and Line nested within Species as a random factor.

2.3.3 Reproductive output

For the fecundity analysis, the model was fitted to a negative binomial probability distribution and a log link. To deal with overdispersion, zero-inflated modeling was incorporated (Zuur et al, 2013). For the fertility analysis, the model was fitted to a binary response using a generalized model with a Bernoulli probability distribution. We use a binary fertile/infertile measure rather than counting larvae because our methods were likely to result in many sterile vials, producing a dataset of offspring counts with many zeros. Quantitative models typically have difficulty with such data.

3. Results

3.1 Lifespan and survival

On average, flies' lifespan under dormancy-inducing conditions was significantly longer ($\chi^2_{(1)}$=698.65, p<10^{-9}) than in the control group kept at 25°C (Fig. 2, Table S2). The model also showed a significant Species-by-Acclimation Condition-by-Mating Status interaction ($\chi^2_{(1)}$= 8.28, p=0.004; Table 1). *A posteriori* comparisons revealed three different groups (Fig. 2). *D. buzzatii* virgin flies had the longest lifespan (averaging 102 days) as compared to the rest of the groups under dormancy-inducing conditions, which included mated *D. buzzatii* males and females, and virgin and mated *D. koepferae* of both sexes (with an average of 71 days). Finally, all groups kept under control conditions exhibited the shortest lifespan (an average of 25 days), irrespective of mating status and species.

GAMM and COXME analyses indicated that fly survival varied significantly across species, sexes (only in COXME analysis), acclimation conditions and mating status (Fig. 3, Table 2). As observed in the lifespan analysis, flies kept under dormancy-inducing conditions had a significantly increased probability of survival in comparison to the control flies (Fig. S2). Survival analysis involving only groups under dormancy-inducing conditions showed that females survived longer than males, and a significant interaction between species and mating status factors (Table S3). *A posteriori* comparisons indicated that virgin *D. buzzatii* flies had a significantly higher chance of survival than the rest of the groups (Fig. S2, Table S4).

3.2 Ovarian Maturation

We investigated sexual maturity of virgin females along the long-term cold-exposure assay. Only 46 out of the 1420 dissected females showed vitellogenic ovaries, meaning that most of the virgin females (97%) arrested their ovarian maturation. We found a significant Age-by-Status interaction (Table S5), as only within the group of naturally dead females, the incidence of ovarian maturation significantly increased with time ($\chi^2_{(1)}$=7.66, p=0.0057, see Table S5). The analysis also indicated that *D. buzzatii* had a significantly higher proportion of females with vitellogenic ovaries than *D. koepferae* ($\chi^2_{(1)}$=4.79, p=0.0286).

3.3 Reproductive Output

3.3.1 Fecundity

The model showed significant differences for all main factors (Table 3). *D. buzzatii* had a significantly higher fecundity (63.6 eggs/female) across all treatments ($\chi^2_{(1)}$=13.2, p=0.0003) than *D. koepferae* (28.2 eggs/female). Also, fecundity differences across reproductive statuses were highly significant ($\chi^2_{(4)}$=65.47, p=2.05x10^{-13}). Tukey's tests revealed a high and similar fecundity for the groups of Post-Cold Mated Females and the Control (Table 4). In contrast, the rest of the groups showed significantly lower fecundity (Table 4).

3.3.2 Fertility

The fertility model showed significant differences for all main factors (Table 5). Overall, *D. buzzatii* showed a significantly ($\chi^2_{(1)}$= 4.11, p = 0.0427) higher fertility than *D. koepferae* (29.6% and 23.1%, respectively). Likewise, differences across reproductive groups were highly significant ($\chi^2_{(1)}$= 82.82, p <10^{-9}). Fertility in Post-Cold Mated Females and the Control was higher than in the rest of the groups

(Figure 4, Table S6), suggesting that only females that mated after long-term cold-exposure were insensitive to fertility loss.

4. Discussion

In the present study we investigate how low temperature acclimation affects lifespan and reproduction, searching for possible overwintering strategies in the pair of sibling species *D. buzzatii* and *D. koepferae*. In ectotherms like fruit flies, low temperatures may be expected to exert beneficial effects on survival, as enzymatic reactions and metabolic processes decelerate. Long-term cold-exposure, however, can also impose detrimental effects (e.g. cold injuries in somatic or reproductive tissues). Below, we will discuss positive and negative effects of long-term cold-exposure on several fitness-related traits.

4.1 Cold-induced lifespan extension

Cold-acclimation induced a remarkable lifespan extension in both species, a fact that indicates a slowed senescence during reproductive dormancy. Under dormancy-inducing conditions, *D. buzzatii* outlived its sibling species *D. koepferae*. Mean lifespan was 94 days in *D. buzzatii*, and 67 days in *D. koepferae*, times that exceed the period in which temperature is below 10°C in the collecting site (Table S1). These results suggest that both cactophilic species are capable of surviving winter as adults, with males and females living long enough to contribute to the population build-up of the following season. Mortality was generally low during the first 42 days of cold exposure (Fig. 3), with only 7.7% of the flies dying during the period.

Nonetheless, our results indicate that other factors besides lowered senescence are at play, as survival and longevity under cold-exposure also depend on the interplay between the species and mating status, a fact that was supported by both GAMM and COXME analyses for survival (Table 2) and the GLMM analysis for longevity

(Table 1). On the one hand, virgin and mated *D. koepferae* flies exhibited similar lifespans and mortality rates, as it was also reported for winter-acclimated *D. suzukii* (Panel et al., 2020). On the other hand, virgin *D. buzzatii* flies have a higher probability of daily survival than their mated counterparts, suggesting a survival cost of reproduction in this species (Flatt, 2011). As a consequence, virgin *D. buzzatii* flies were the ones that had the longest lifespans (Fig. 2), suggesting a selective advantage of unmated flies during population build-up in this species.

4.2 Lack of ovarian maturation in virgin females maintained under winter-like conditions

In a previous paper, we showed that reproductive maturation could be suppressed in *D. buzzatii* and *D. koepferae* by maintaining females at 10ºC and 10:14 L:D for at least one month, in a form of reversible reproductive dormancy (Mensch et al., 2017). The present results show that reproductive arrest lasted the entire lifespan, as virtually no ovarian maturation occurred under winter-like conditions in virgin females of both species. Under dormancy-inducing conditions, energy seems to be mainly allocated to survival rather than reproduction. Indeed, only within the group of naturally dead females, the incidence of ovarian maturation (*i.e.*, vitellogenic ovaries) significantly increased with time, a fact that may reflect a survival cost of reproduction (Flatt, 2011). However, as reproductive dormancy is a reversible state, when virgin (and immature) females of both species were transferred to 25°C for one week, they reached sexual maturity, mated and laid eggs, reaching a similar fecundity as the control kept at 25°C, which could be envisaged as the optimum condition for egg production. In contrast, when males were cold-exposed, it resulted in a reduced fecundity (Table 4).

366

367 **4.3 Males are more susceptible to cold-induced fertility loss than females**

368 Fertility analyses provided one of the most striking differences among experimental
369 groups. While females that had mated after cold-exposure (Post-cold mated
370 females) exhibited similar hatching rates as the non-acclimated control group, the
371 rest of the groups (including the post-cold mated males) exhibited large reduced
372 fertility after cold-exposure. These results point out that the reduction of fertility of
373 males mated after cold-exposure is higher than that of post-cold mated females. In
374 addition, females were poor at protecting mature sperm inside their reproductive
375 tract; while pre-cold mated flies of *D. buzzatii* showed low fertility, pre-cold mated *D.*
376 *koepferae* females were not able to produce any offspring. These two facts suggest
377 that cold treatment compromises spermatogenesis and reduces the viability of
378 mature sperm, particularly in *D. koepferae*. Interestingly, a small proportion of cold-
379 treated *D. koepferae* males were impaired to exhibit adequate courtship behaviour,
380 manifested in erratic locomotor activity (data not shown). Overall, our findings
381 indicate that reduced male fertility was responsible for the low fertility of post-cold
382 mated flies, and suggest that male fertility thermal limits could account for the
383 temporal window for reproductive season in temperate regions. Again, even if fertility
384 of post-cold mated flies was extremely low in *D. buzzatii*, cold temperature likely
385 sterilized *D. koepferae* males, indicating that cold carry-over effects are stronger for
386 the species with the shorter lifespan. The suppression of hatching (*i.e.* viable eggs)
387 observed in *D. koepferae* calls for attention, as isofemale lines used of both species
388 derived from the same location. In the southernmost populations of *D. buzzatii* and in
389 a large fraction of *D. koepferae* distribution range, including Valle Fértil, the
390 environmental conditions are similar to the winter-like conditions settled in the

present study (see Table S1). The fact that cold sterilized *D. koepferae* males suggest a faster reproductive senescence compared to its sibling species *D. buzzatii*. Accordingly, females of *D. koepferae* usually lay large amounts of eggs soon after mating, and probably before new matings, whereas *D. buzzatii* females lay eggs for a longer time, during which chances of remating are higher than in its sibling (Fanara et al., 1999; Fanara & Hasson, 2001; Hurtado et al., 2013), a likely behavioral adaptation for a short-lived species. In effect, it is important to mention that our experimental design only involved a single mating before or after cold-exposure. Fertility would have probably increased with a higher number of crossings with different males (*i.e.* larger number of sperm). Also, our experimental setting did not involve temperature fluctuations, which may have favored fertility recovery after cold-exposure, by taking advantage of the daily warm pulses, even with the same average low temperature (Colinet et al., 2015). Further studies are needed to better understand the mechanisms preventing cold damage in more ecologically relevant scenarios (*i.e.* including thermal fluctuations).

4.4 Ecophysiology and evolution of overwintering in cactophilic *Drosophila*

Our results showed that both cactophilic species are capable of surviving the winter as adults. However, while females that mate after cold-exposure (Post-cold mated females) maintain high fertility, males exhibit high incidence of sterility after dormancy. As a consequence, it is possible that a fraction of the population overwinters as pre-adult stages. Eggs laid in autumn could survive winter inside decomposing host plants and take advantage of daily thermal fluctuations. Lower temperatures surely slow down larval development (Folguera et al., 2008) and stretch it up until spring. This may explain, at least in part, the winter persistence and population build-up of *D. koepferae*, but also of *D. buzzatii* whose reproductive

output is challenged by cold exposure at dormancy-inducing conditions as we showed here. We cannot rule out, however, that microclimates inside decomposing cacti can also serve as shelters for adults during extreme conditions. In any case, as *D. buzzatii* and *D. koepferae* are closely related members of a clade of tropical origin (Ruiz & Wasserman, 1993) it is intriguing to find that females of long-lived *D. buzzatii* are capable of protecting stored sperm from cold temperature damage and produce viable progeny when thermal conditions are restored. Though in terms of their chill-coma recovery times and Critical Thermal Minima (CT_{min}) *D. buzzatii* and *D. koepferae* showed similar cold tolerance (Mensch et al., 2017; Mensch et al., 2021), only the former species partially recover fertility after cold exposure within our assay period. Taken into account that both species co-exist in most of the distribution range of *D. koepferae*, such differentiation of their fundamental thermal niches can result in early population build-up by *D. buzzatii.* This may contribute to its faster population growth during the early season in temperate sympatric regions. In effect, thermal limits on reproductive ability are possibly more relevant factors to consider than limits on survival and thermal tolerances when estimating population dynamics and species distributions, as it has been shown for high temperatures (David et al., 2005; Jørgensen et al., 2006; Parratt et al 2021). As a consequence, the reproductive ability after cold exposure may account, at least in part, for the differential success of *D. buzzatii* in colonizing a wider distribution range.

From an evolutionary standpoint, as *D. buzzatii* and *D. koepferae* are sibling species, our findings suggest that low-thermal fertility limits have expanded in the *D. buzzatii* lineage. Protecting stored sperm from cold damage (in females) and maintaining spermatogenesis and seminal fluids after cold exposure (in males) could explain the ability of *D. buzzatii* to partially recover fertility and expands the low thermal fertility

limit. Further studies should address which components of the male's reproductive system (e.g. testes, accessory glands, seminal vesicle) are compromised at low temperatures and could account for partial and complete fertility loss, in *D. buzzatii* and *D.koepferae,* respectively.

5. Funding

This work was financially supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad de Buenos Aires and Agencia Nacional de Promoción Científica y Tecnológica to J.M. and E.H. and with scholarships granted to L.E.K (University of Buenos Aires) and to F.P. (CONICET).

6. Declaration of Interest

None

7. Acknowledgement

We thank the Associate Editor, Dr. Glenn Tattersall, and the three anonymous reviewers for constructive comments on an earlier version of the manuscript. We also would like to acknowledge Dr. Nicolás Flaibani and Dr. Carmen Rolandi for their collaboration in the initial phase of statistical analyses. We like to thank Dr. Eduardo M. Soto for providing the stock flies used in this study and Nina Saroka for her collaboration in some experiments.

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Conceptualization: JM

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Figures and Tables

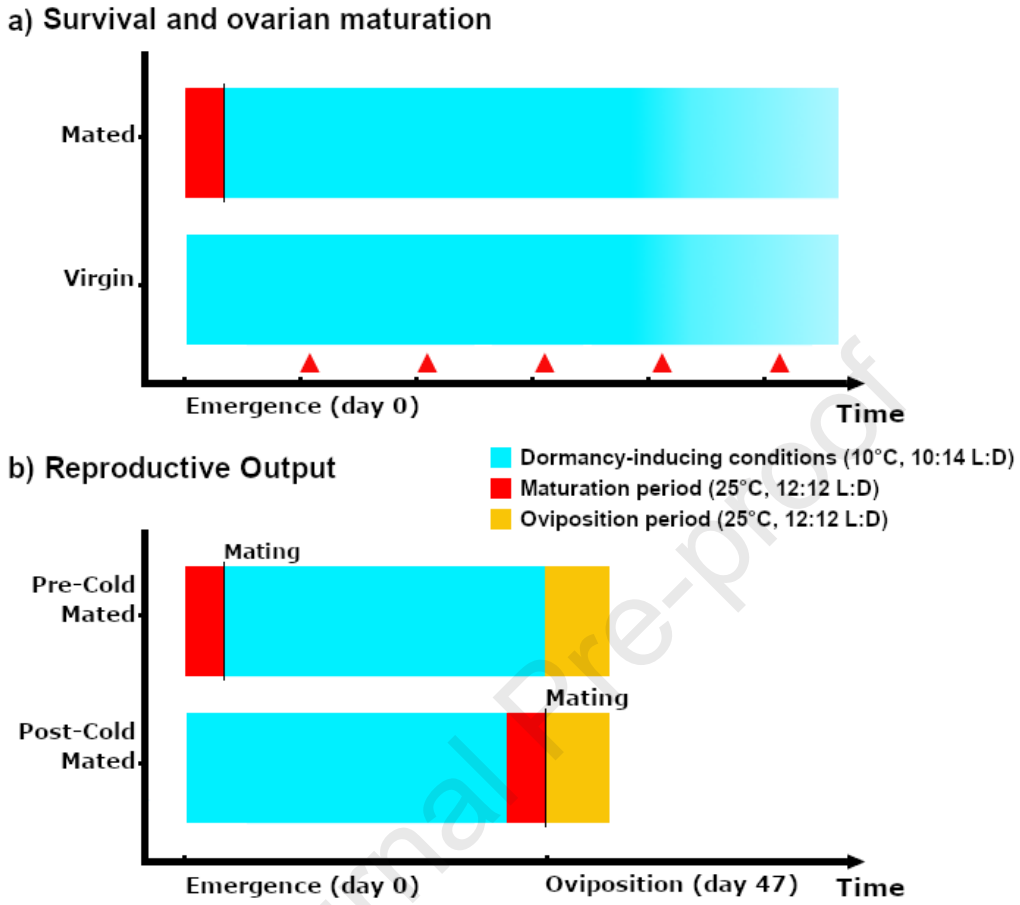

Figure 1. Experimental design. **A**: Survival assay: survival was measured in mated and virgin flies kept at dormancy-inducing conditions. To score ovarian maturation random females from the virgin group were dissected every 15 days (red triangles), as well as dead females resulting from the survival assay. **B:** Reproductive output assay: flies were either mated at 25°C and then placed at dormancy-inducing conditions (Pre-Cold Mated) or previously exposed to the cold treatment and then allowed to copulate (Post-Cold Mated).

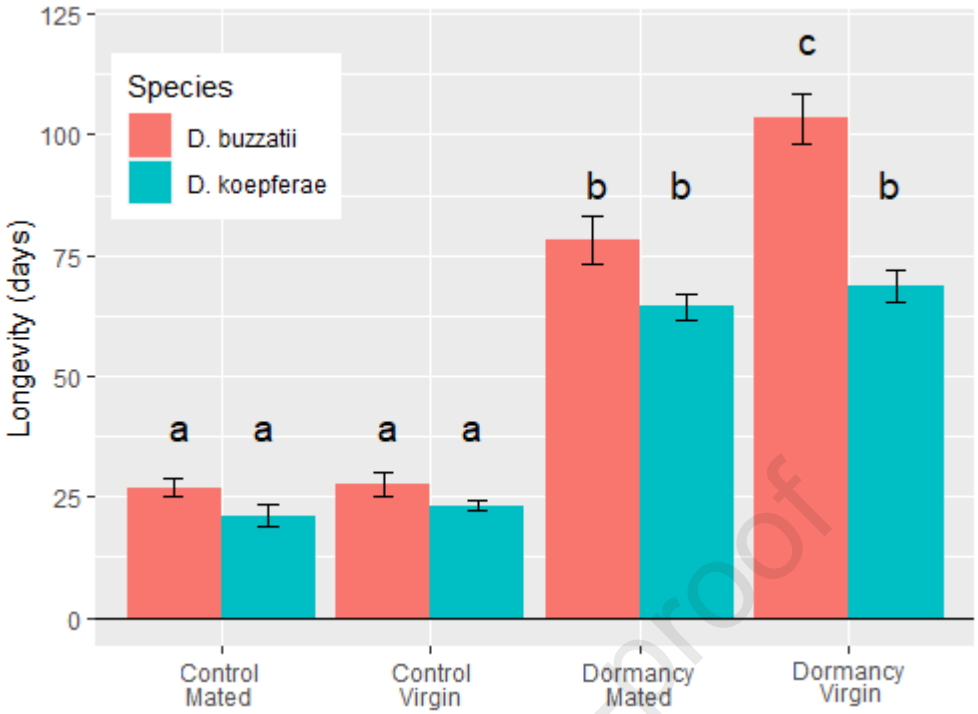

Figure 2. Longevity, expressed in days, for the different species and mating status groups at dormancy-inducing conditions and control. Bars represent mean longevity value, error bars represent standard deviation. Different letters indicate significant differences in Tukey's tests ($P < 0.05$).

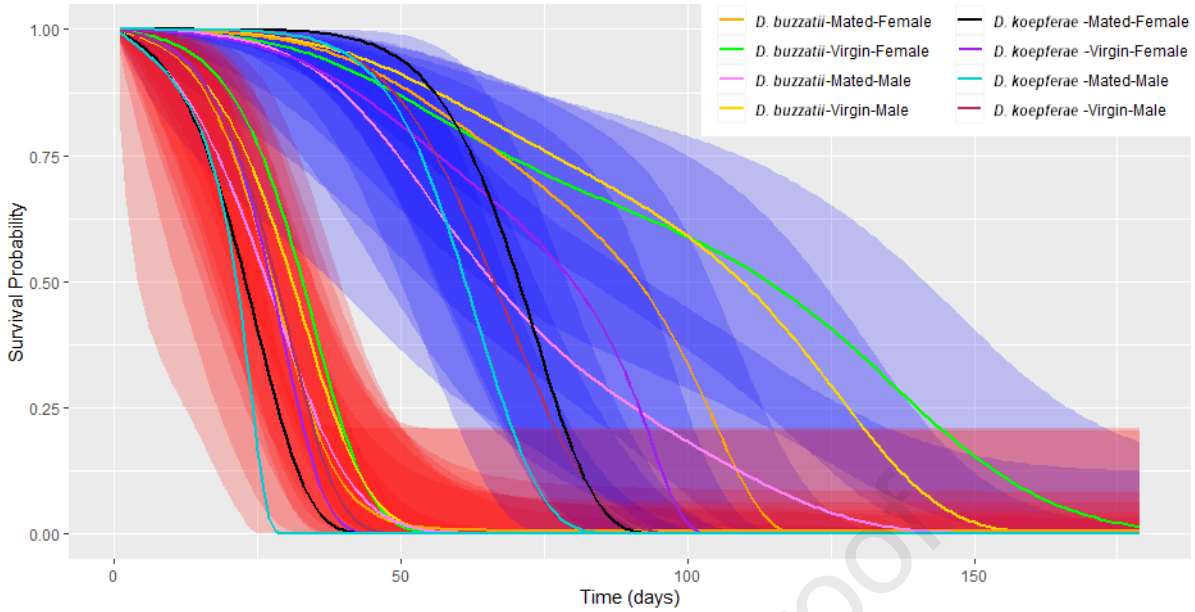

Figure 3. Survival functions of all experimental groups (8 colors) produced by GAMM analyses. Groups under dormancy-inducing conditions and control conditions are shown in blue and red shades, respectively. Lines indicate the survival functions and shades encompass 95% CI. GAMM analyses indicated that fly survival varied significantly between species, sexes, acclimation conditions and mating status.

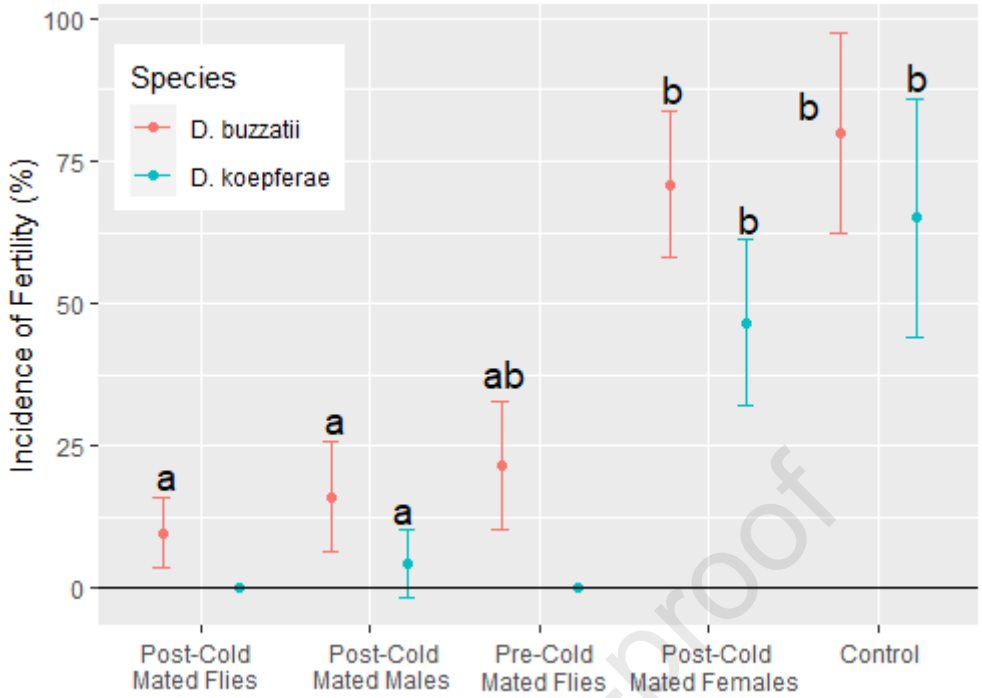

Figure 4. Incidence of Fertility by reproductive status and species. Bars represent 95% confidence intervals, points represent mean fertility. Different letters indicate significant differences in Tukey's tests ($P < 0.05$).

Variable	χ^2	Df	p-Value
Mating Status	4.42	1	**0.0354**
Species	3.52	1	0.0605
Sex	5.00	1	**0.0253**
Temperature	698.65	1	**$1<10^{-9}$**
Mating Status:Species	1.01	1	0.3144
Mating Status:Sex	0.08	1	0.7730
Specie:Sex	0.03	1	0.8586
Mating Status:Temperature	6.82	1	**0.0090**
Species:Temperature	19.92	1	**$8.1x10^{-6}$**
Sex:Temperature	3.08	1	0.0791
Mating Status:Species:Sex	0.01	1	0.9303
Mating Status:Species:Temperature	8.28	1	**0.0040**
Mating status:Sex:Temperature	0.10	1	0.7551
Species:Sex:Temperature	0.09	1	0.7627
Mating Status:Species:Temperature:Sex	0.30	1	0.5850

Table 1. Analysis of Deviance (Type II Wald χ^2 Test) for Longevity. Bold values denote statistical significance at the $p < 0.05$ level.

726

Effect	COXME		GAMM	
	χ^2	p-Value	χ^2	p-Value
Acclimation condition	162.56	$<1x10^{-9}$	94.95	$<\mathbf{1x10^{-9}}$
Species	5.51	0.0189	1.82	**0.0039**
Sex	5.20	0.0226	8.31	0.1778
Mating status	26.97	$2.1x10^{-7}$	13.97	**0.0002**
Acclimation condition:Species	3.49	0.0617	0.73	**0.0391**
Acclimation condition:Sex	1.25	0.2644	4.26	0.3937
Species:Sex	0.01	0.9435	0.11	0.7426
Acclimation condition:Mating status	2.76	0.0966	3.74	0.0532
Species:Mating status	3.57	0.0589	0.05	0.8214
Sex:Mating status	0.21	0.6448	2.46	0.1170
Acclimation condition:Species:Sex	0.06	0.8065	0.07	0.7912
Acclimation condition:Species:Mating status	5.91	0.0151	0.06	0.2236
Acclimation condition:Sex:Mating status	0.43	0.5137	1.48	0.8107
Species:Sex:Mating status	0.06	0.8091	0.36	0.5487
Acclimation condition:Species:Sex:Mating status	0.58	0.4468	0.35	0.5530
Random Effect				
	σ	**σ^2**	**χ^2**	**p-Value**
Line	0.52	0.27	35.35	$\mathbf{<10^{-9}}$

727

728 **Table 2.** Analyses of Variance performed over both COXME and GAMM models. For the random
729 effect, σ and σ^2 represent variance and standard deviation respectively. Bold values denote statistical
730 significance at the $p < 0.05$ level.

Variable	χ^2	Df	p-Value
Reproductive Status	65.47	4	**$<10^{-9}$**
Species	13.20	1	**0.0003**
Species:Reproductive Status	6.20	4	0.1850

Table 3. Analysis of Deviance (Type II Wald Test) for Fecundity. Bold values denote statistical significance at the $p < 0.05$ level.

Reproductive Status	Species	Mean	SD	N
Post-Cold Mated Females	*D. buzzatii*	140.98	130.98	48
Post-Cold Mated Females	*D. koepferae*	59.5	80.76	45
Control	*D. buzzatii*	155.9	100.85	20
Control	*D. koepferae*	33.9	38.50	20
Post-Cold Mated Flies	*D. buzzatii*	37.2	56.69	92
Post-Cold Mated Flies	*D. koepferae*	7.9	18.69	22
Pre-Cold Mated Flies	*D. buzzatii*	20.5	34.19	51
Pre-Cold Mated Flies	*D. koepferae*	1.08	5.31	24
Post-Cold Mated Males	*D. buzzatii*	46.82	80.45	56
Post-Cold Mated Males	*D. koepferae*	18.82	32.61	45

Table 4. Mean fecundity of each experimental group. SD stands: standard deviation. N: sample size.

Variable	χ^2	Df	p-Value
Reproductive Status	82.82	4	**$<10^{-9}$**
Species	4.11	1	**0.0427**
Species:Reproductive Status	0.41	4	0.9819

Table 5. Analysis of Deviance (Type II Wald Test) for Fertility. Bold values denote statistical significance at the $p < 0.05$ level.

Highlights

* Cold-acclimation induced a remarkable lifespan extension in both species.

* Ovarian maturation was almost completely halted during reproductive dormancy.

* Males are more susceptible to cold-induced fertility loss than females

* Only females of *D. buzzatii* were capable of protecting stored sperm from cold damage and producing viable progeny.

Funding

This work was financially supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad de Buenos Aires and Agencia Nacional de Promoción Científica y Tecnológica to J.M. and E.H. and with scholarships granted to L.E.K (University of Buenos Aires) and to F.P. (CONICET).

Declaration of Interest

None