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INTERACTIONS BETWEEN ENVIRONMENTAL STRESS AND MALE MATING SUCCESS MAY ENHANCE EVOLUTIONARY DIVERGENCE OF STRESS-RESISTANT *DROSOPHILA* POPULATIONS

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Adaptation of natural and laboratory-selected populations of *Drosophila* to desiccation stress results in enhanced water conservation abilities, and thus increased stress resistance. In this study, we tested whether laboratory selection for desiccation resistance is also reflected in increased mating success of adapted *D. melanogaster* males under desiccating conditions. Adapted flies perform better under stressful conditions, and as expected males from desiccation-selected populations exhibited significantly higher relative mating success in comparison with controls after 5–6 h of desiccation. However, we show evidence for a trade-off between survival under stressful conditions and mating success in nonstressful and even mildly stressful environments (2.5–3 h of desiccation), where males from selected populations were involved in only ~40% of observed copulations. This suggests that mutations favored by natural selection, associated with survival when resources are limited, may only be favored by sexual selection above a minimal "threshold" stress level. At milder stress levels increased resistance comes at a cost of lower relative mating success, and thus reduced fitness. This interaction between stress and relative male mating success of adapted and nonadapted males could interrupt gene flow, thus facilitating divergence of resistant populations from the ancestral population.

KEY WORDS: **Desiccation resistance, *Drosophila melanogaster*, evolutionary divergence, male mating success.**

Desiccation resistance in both natural and laboratory-selected populations in the genus *Drosophila* has been extensively studied (reviewed by Hoffmann and Harshman 1999; Gibbs 2002). In natural populations, mesic species were found to be more susceptible to desiccation in comparison with species from more arid regions (van Herrewege and David 1997; Gibbs and Matzkin 2001; Gibbs et al. 2003). A similar pattern of geographical variation has been reported in most within-species studies, where desiccation resistance has been shown to be correlated with environmental conditions (reviewed by Hoffmann and Harshman 1999, but see Matzkin et al. 2007). Laboratory selection of *Drosophila melanogaster* results in a significant increase in desiccation resistance (e.g., Gibbs et al. 1997; Chippindale et al. 1998). Desiccation resistance in laboratory populations of *D. melanogaster* is associated with reduced preadult viability

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(Chippindale et al. 1998; but see Hoffmann and Parsons 1993) and increased longevity (see Hoffmann and Harshman 1999). However, evidence for trade-offs between longevity and fecundity suggests contrasting effects of selection for stress resistance on overall reproductive output (Prasad and Joshi 2003 and references therein).

Although the effects of stress resistance on survival and fecundity are well documented, there is little experimental evidence regarding the effect of selection for stress resistance on male mating success. Hoffmann and Parsons (1993) did not observe a deviation from a 1:1 mating ratio between desiccation-selected and control *D. melanogaster* under nonstressful conditions. In contrast, laboratory populations of *D. melanogaster* maintained under different temperature and humidity conditions showed environment-dependent, largely premating, sexual isolation (Kilias et al. 1980). Similarly, adaptation of *D. pseudoobscura* populations to two different stressful media resulted in behavioral isolation and nonrandom mating as a by-product (Dodd 1989). It has also been shown that thermal adaptation to experimental assay conditions increases male mating success in *D. melanogaster* (Dolgin et al. 2006). Interestingly, flies from opposite slopes of the "Evolution Canyon" in Israel, characterized by different microclimatic conditions, exhibited not only significantly different desiccation resistance (Nevo et al. 1998) but also mating preference for flies from the same slope, despite the relatively short dispersal distances (100–400 m) between the south- and north-facing slopes (Korol et al. 2000).

Correlations between natural and sexual selection could lead to population divergence. As premating isolation is often associated with the initial stages of evolutionary divergence (Coyne 1992), we hypothesized that higher mating success of resistant males under stressful conditions could indicate an underlying mechanism for divergence of desiccation-resistant populations. The goal of this study was to test whether selection for desiccation resistance, resulting in better performance under stressful conditions, is also reflected in higher relative male mating success. We compared mating success rates of selected and control males over a set of three experimental desiccation stress levels, that may be analogous to the increasing stress levels as resources gradually become sparse at the margins of distribution of natural populations.

It has been shown that higher desiccation resistance among *Drosophila* species is associated with lower activity patterns under stressful conditions (Gibbs et al. 2003). We therefore measured metabolic rates of selected and control males under the respective experimental desiccation treatments to verify that courtship activity and copulation during mating assays do not simply reflect differing activity levels resulting in varying encounter rates between males and females.

Materials and Methods

FLY SELECTION AND MAINTENANCE

All populations were maintained at 24.5°C under constant light. We used a fly population founded from ∼400 females collected in New Jersey in 1999. Flies were maintained as a large outbred population in the laboratory until selection began. To minimize the possibility of artifacts due to adaptation to a new environment, the populations were maintained on a standard 3-week stock cycle for 12 generations before selection was started (Chippindale 2006). Pre-adult stages were reared at densities of ∼60 larvae in vials containing 10 mL of corn meal–sucrose–yeast medium. After 2 weeks, adult flies (approximately four days posteclosion) were transferred to 5.5 L Plexiglas population cages containing two petri dishes of food. A cloth sleeve covered one end and allowed access to the cage. The medium was changed every two days. After four days, yeast paste was added to stimulate egg production. Approximately 1200 eggs were collected after seven days to found the next generation.

Selection for desiccation resistance was performed by removing food from the cages 1–4 h after transferring the flies. A cheesecloth-covered dish containing ∼200 g of silica gel desiccant was placed inside, and the open end of the cage was loosely covered with plastic wrap to allow gas exchange while reducing influx of water vapor from the surroundings. Initially, the cages contained ∼7500 flies. They were checked hourly until 80–85% of the flies had died. The desiccant was then removed and fresh food was provided to the survivors. The flies were given several days to recover before egg collection for the next generation. Population sizes in all treatments were maintained to provide an estimated 1000–1500 adult population after selection. Flies were selected as described above for the first 30 generations following the initiation of selection. This was followed by ∼60 generations of less severe selection before the experiments were carried out. During this time desiccation resistance was maintained by subjecting flies to desiccation for 24 h after transferring them to the cages, a treatment that kills nearly all control flies (A. G. Gibbs, pers. obs.). It has been shown previously that desiccation resistance of similar populations was not compromised after 35 generations of relaxed selection (Passananti et al. 2004). Three replicate populations (D_A-D_C, F_A- F_C), sharing a common ancestry, were maintained from each of the selected (D) and control (F) populations, as was the common ancestral population (Ter), which like the control populations was provided with food plates during the desiccation stress exposure of D populations. The mating trials were conducted after a total of 88–95 generations of fly selection.

Fly populations were taken off selection for one generation prior to the assays to avoid parental effects. Parental generation flies were placed in 175-mL bottles with 50-mL cornmeal food

and yeast paste was added to stimulate egg production. Egg collection was carried out by transferring the flies to empty bottles that were covered with a 35 × 10 mm plate containing grape agar as a substrate for egg laying. Sets of 70–80 eggs were then placed in food vials containing approximately 10 mL of cornmeal food, and incubated at 24.5°C and constant light.

MATING ASSAYS

We used a modification of the mating assay protocol reported by Dolgin et al. (2006). Selected and control males were paired with females from their ancestral population, to rule out potential effects of male–female coevolution within populations. Mating assays were conducted with adult flies at four days posteclosion, and started by placing 10 virgin Ter females and 15 males of each selection treatment (D and F) in Plexiglas cages. Virgin Ter females were obtained by sexing third instar and wandering larvae, when differences in gonad appearance are distinguishable through the integument (Folk et al. 2001). Sets of 10 female larvae were kept in vials with fresh food, and the vials were examined again at two days posteclosion for confirmation of male exclusion. Vials containing males were discarded. Sets of less than 10 flies due to larval mortality were complemented and replaced in fresh food vials until the assays.

Flies from the six D and F populations were kept in the original larval vials until two days posteclosion, and then sexed. Sets of 15 males were placed in vials with fresh dyed (red or blue food coloring) cornmeal food for two days for later male identification. Food color treatment was alternated to rule out a color effect.

Three levels of desiccation stress were included in this study–control treatment, where flies were placed in the assay cages directly from the food vials (0 h), and two levels of desiccation stress where assays followed desiccation of male D and F flies for 2.5 h or 5 h at room temperature (23–25°C). Virgin Ter females were always kept in food vials until the initiation of the assays. For desiccation treatments males were knocked out by brief exposure to CO_2, transferred to empty vials and restricted to the lower half of the vials with a foam stopper. Silica gel was then added above the stopper to maintain low humidity, and the vial was sealed with parafilm. All flies recovered from the brief anesthesia within seconds.

The mating assays were conducted at room temperature, and started by placing the flies in 23 × 19 × 13 cm transparent Plexiglas cages with a cloth sleeve covering one side, allowing access to the cage interior. Copulation in *D. melanogaster* lasts ∼20 min (Ashburner 2005), and therefore the cages were checked every 10 min and mating couples were aspirated out and placed in empty vials for subsequent identification of the males. No mortality was observed in the control populations prior to 7 h of desiccation during preliminary tests, and therefore experimental desiccation levels were set to 2.5 and 5 h prior to initiation of the assays. During the 75-min-long mating assays the flies had no access to food or water, and ambient humidity was low (typically 15–30%), so the flies were stressed for a total of ∼2.5–3.5 and 5–6 h, respectively.

RESPIROMETRY

Rates of CO_2 output were measured at 25°C by placing groups of 9–15 male flies in 5-mL glass-aluminum chambers after each of the desiccation treatments (as described above), and pumping CO_2-free dry air through the chamber and into a Li-Cor (Lincoln, NE) LI-6262 infrared CO_2 analyzer. The flies were acclimated to the chamber and airflow for 15 min prior to the 15-min-long recording. This corresponds to desiccation stress levels equivalent to those experienced by the flies during the first half of the mating assays, during which the majority of copulation events were observed. Rates of CO_2 output were measured for four groups of flies from each of the six populations at each of the three desiccation levels.

STATISTICS

Mating assay data were analyzed by replicated goodness-of-fit *G*-tests (Sokal and Rohlf 1995). Data from individual cages were pooled for six replications for each desiccation stress level, consisting of alternated color treatment for each of three replicate populations from each selection population.

Respirometry measurements were analyzed by analysis of variance (ANOVA) using Statsoft (Tulsa, OK) Statistica for Windows version 7.0. No significant differences were found between repeats (ANOVA; $F_{3,72} = 0.430$, $P = 0.74$), and therefore results were pooled to a three-way mixed model ANOVA with selection treatment (S; fixed effect), replicate population (R; random effect) and desiccation time (T; fixed effect) as main factors.

Results

MATING ASSAYS

Mating assays were carried out until the number of aspirated mating couples was around 250 for each desiccation treatment. In total, 249, 264, and 240 copulations were observed at the 0, 2.5, and 5 h desiccation treatments, respectively. As expected, mating frequencies declined with desiccation stress, with 69% (249/360) of the females mating at 0 h, 40% (264/660) at 2.5 h and 31% (240/780) at 5 h desiccation. This corresponded to an average number of mating couples per cage among replicate populations ranging from 6.3 to 7.4 at 0 h, 3.8 to 4.3 at 2.5 h and 2.9 to 3.3 at 5 h desiccation. Only two incidents of mortality from a total of 5400 males were observed in the cages throughout the assays, and were therefore considered negligible for statistical analysis. From a total of 753 mating males, 376 (49.9%) were reared on

Table 1. Total number of selected (D) and control (F) mating males, from three replicate populations and two food color treatments, at the three desiccation stress levels. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Replicate population	Color of D males	0		2.5		5	
		D	F	D	F	D	F
A	Blue	21	27	17	26	29	17
A	Red	17	20	20	32	31	10
B	Blue	16	27	16	29	24	15
B	Red	17	29	14	25	18	20
C	Blue	20	22	16	22	18	15
C	Red	14	19	22	25	28	15
Total		105	144	105	159	148	92
Total number of males from each population		540		990		1170	
Total number of females		360		660		780	
$G_{heterogeneity(5df)}$		1.73[1]		1.67[1]		7.73[1]	
$G_{pooled(1df)}$		6.13*		11.12***		13.19***	

[1]Nonsignificant heterogeneity among six replicate D:F comparisons within each desiccation treatment. Asterisks denote significant differences of pooled data from the expected 1:1 ratio (see text).

red-dyed food. The effect of dye color on mating success was not significant in any of the trials (pooled *G*-tests, $P = 0.61$–0.95). Therefore data from individual cages were pooled for six replications for each desiccation stress level, consisting of alternated color treatment for each of three replicate populations from each selection population.

Significant differences in mating success of selected (D) and control (F) males were observed in all three experimental desiccation stress levels. At the 0 and 2.5 h treatments only 40–42% of mating males were from the desiccation-selected populations, with the figure increasing to 62% after the 5 h desiccation treatment. Heterogeneity *G*-tests (G_H) indicated no significant differences in D:F mating success ratios among the six replications within any of the desiccation treatments, with *P*-values of 0.89, 0.89, and 0.17 for calculated G_H at 0, 2.5, and 5 h of desiccation, respectively (Table 1). The data for all six replicated comparisons were pooled yielding statistically significant differences in mating success of D and F males in all three desiccation treatments (calculated pooled G, G_P; see Table 1).

RESPIROMETRY

Respirometry was performed to test whether observed differences and changes in mating success under stressful conditions were correlated with changes in activity of selected and control males during desiccation stress, as reflected in their metabolic rates (MR, as indicated by CO_2 output). Results show significant effects of selection treatment and desiccation time, as well as interaction of the two factors, on metabolic rates (Table 2). No significant difference was found between MR of nondesiccated D and F males, as measured within 30 min of transfer from the food vials ($P = 0.13$; $t_{22} = 1.55$) (Fig. 1). However, MR of F males remained unchanged throughout the range of desiccation levels, whereas D males exhibited a significant decrease in metabolic rates after 2.5 h of desiccation compared to initial levels (Fig. 1). This meant that metabolic rates of desiccated D males were significantly lower than those recorded for F males after 2.5 h ($P < 0.001$; $t_{22} = 5.13$) and 5 h ($P < 0.001$; $t_{22} = 7.84$) of desiccation (Fig. 1).

Table 2. Analysis of variance (ANOVA) for the effects of selection treatment, replicate population, and desiccation time on metabolic rate of male flies, expressed as CO_2 output. *$P < 0.05$; **$P < 0.01$.

Effect	df	MS[1]	*F*	*P*
Selection (*S*)	1	15.82 (0.49)	32.25	0.0296*
Replicate(*R*)	2	0.26 (0.39)	0.66	0.6412
Time (*T*)	2	3.36 (0.08)	40.05	0.0022**
$S \times R$	2	0.49 (0.18)	2.68	0.1826
$S \times T$	2	2.58 (0.18)	14.10	0.0154*
$R \times T$	4	0.08 (0.18)	0.46	0.7654
$S \times R \times T$	4	0.18 (0.26)	0.70	0.5989
Error	54	0.26		

[1]Values in brackets indicate denominator for each test.

Discussion

Increased desiccation resistance in *Drosophila*, expressed as longer adult survival time under stressful conditions, has been consistently reported in both laboratory-selected populations and natural populations adapted to arid and semi-arid habitats

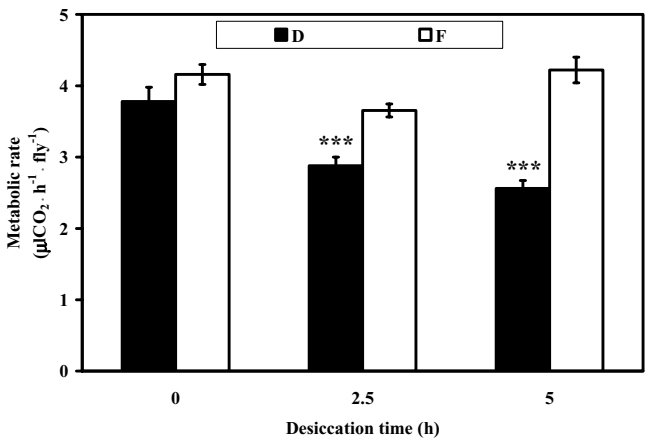

Figure 1. Mean (± SE) CO_2 output rates of selected (D) and control (F) males after 0, 2.5, and 5 h of desiccation. Asterisks denote significant differences from values at 0 h of desiccation ($P < 0.001$).

(e.g., van Herrewege and David 1997; Hoffman and Harshman 1999; Gibbs and Matzkin 2001; Gibbs and Gefen 2009; but see Matzkin et al. 2007). It was therefore reasonable to expect that males from populations adapted to desiccation stress would perform better and be more successful than their controls in mating under stressful desiccating conditions.

A greater number of assays were required to reach the target of 250 copulations with increasing desiccation stress levels (Table 1), which demonstrates the decline in mating performance of *Drosophila* under increasing levels of environmental stress (Fasolo and Krebs 2004). The gradual decrease in mating occurrences culminated in ~50% decrease at 5 h desiccation compared to control conditions. As predicted, a significantly higher proportion (62%) of mating males in assays following 5 h of desiccation were from the stress-resistant populations (D). This result is in accordance with a previous mate-choice study that showed higher male mating success in *D. melanogaster* populations thermally adapted to assay temperature compared with males adapted to an alternative temperature (Dolgin et al. 2006). However, while thermal adaptation preserved mutations favored by both natural and sexual selection, we found a significant interaction between the effects of population (selected vs. control) and stress level on relative male mating success. D males had higher mating success following 5 h of desiccation stress, whereas control (F) males had significantly higher relative mating success not only in a nonstressful environment but also after 2.5–3.5 h of desiccation (Table 1).

Our early attempts to carry out mating assays with greater numbers of flies (20 Ter females and 30 males from each assayed population) resulted in mating ratios not different from 1:1 under all three experimental desiccation levels (data not shown). This was in agreement with previously reported values based on high-density mating assays (Hoffmann and Parsons 1993). We feel that the discrepancy from the nonrandom mating patterns reported here was largely a result of interference and coincidental male–female interactions leading to mating in overcrowded assay cages. Nevertheless, spatial and temporal availability of resources could be reflected in fly densities at natural breeding sites (Markow and O'Grady 2008). The density dependence of the observed mating ratios in our laboratory-based assays highlights the caution required when extrapolating these results to natural populations.

Males from desiccation-selected populations are larger in body size compared with controls (Gibbs et al. 1997; Chippindale et al. 1998; Gefen et al. 2006). As larger body size is widely considered to be a competitive advantage (Partridge et al. 1987a,b; Wilkinson 1987; Markow 1988; but see Markow and Ricker 1992), F males were not expected to mate more frequently when unstressed, which was in contrast with our observations. Quantities of cuticular hydrocarbons (HC), thought to be involved in mating behavior of *Drosophila* (Ferveur 2005), are not different between desiccation-selected and control flies (Gibbs et al. 1997) or among wild populations varying in water loss rates (Parkash et al. 2008). However, differences in the HC composition between selected and control males (Gibbs et al. 1997), coupled with similar laboratory maintenance of Ter and F populations (see methods), mean that we cannot rule out possible advantage for F males in mate recognition.

Nevertheless, the significantly higher mating success of F males was maintained following 2.5–3.5 h of desiccation (Table 1), when both size and adaptation to stressful conditions were expected to be reflected in higher relative mating success of D males. Reduced mating rates (Table 1) and MR (Fig. 1) show significant effects of desiccation stress on D males even after 2.5 h, although these resistant males were still outperformed by their controls. Thus, the higher relative mating success of D males appears to be limited to higher stress levels. This lower mating success of D males under milder conditions indicates a trade-off between survival in stressful environments and a significant fitness component when unstressed, which agrees with the notion of reduced fitness of locally adapted populations in ancestral environments (Proulx 1999).

Interestingly, despite the lack of significant heterogeneity among replicate populations we noticed somewhat higher mating success rates of F males from population B at 0 h (63%) in comparison with populations A and C (55%). This pattern was maintained throughout the desiccation stress levels as F_B males were almost as successful in mating after 5 h desiccation as were D_B, whereas F males totaled 31% and 39% of successfully mating males in the A and C populations, respectively (Table 1). This may reflect a possible difference in genetic composition among initially established replicate populations resulting from genetic drift, which contributed to the observed G_H (Table 1). Nevertheless, we feel that the consistent trend of increasing relative D male mating success at the expense of F males that characterizes all three replicate populations highlights the trade-off between stress resistance and male mating success rates under varying environmental conditions. It is worth noting that the experimental design in this study isolates mating preference from possible male–female coevolution by using Ter females sharing the same evolutionary history with both selected and control flies (see methods). In addition, females were not desiccated prior to the assays, and therefore results are also not affected by possible effects of stress on female mating preference (Grace and Shaw 2004).

In our assays mating couples were aspirated out (see methods), and it could be argued that decreasing the number of females in the cages (number of females in the population) resulted in increased competition for females, and thus overestimation of the relative advantage of the more successful male population in each of the experimental environmental conditions. Nevertheless, remating in female *D. melanogaster* occurs after two to five days on

average (Pyle and Gromko 1981; van Vianen and Bijlsma 1993), whereas males of many *Drosophila* species have been shown to be able to inseminate more than one female (Singh and Singh 2000, and references therein). Therefore, our results could even represent a conservative estimate of the actual magnitude of fitness advantage reflected in the relative mating success rates of the respective populations at different levels of desiccation stress.

An interspecific comparison between *Drosophila* species has shown different activity patterns under desiccation conditions. The mesic *D. melanogaster* exhibited relatively high activity throughout the duration of a 6–7 h measurement, whereas the xeric *D. mojavensis* remained inactive for 12 h, before increasing activity for the next 14 h prior to dying (Gibbs et al. 2003). Desiccation resistance of four-day-old D males was reported to be ~20 h (Chippindale et al. 1998), similar to that of *D. mojavensis*. More active flies may encounter each other and initiate mating more often, and thus mating success and metabolic rates (MR) should be positively correlated. Therefore, if the observed mating success rates of the D and F males were a simple reflection of the effect of the experimental desiccation stress on activity levels, we would expect correlated responses of mating success and MR with changing levels of desiccation stress. Instead, the relatively constant MR of F males throughout the range of experimental desiccation conditions were coupled with a continuous decrease in male mating success rates, from 27% (144/540) successfully mating F males when unstressed to 8% (92/1170) following 5 h of desiccation. Desiccation-selected males exhibited lower metabolic rates as stress progressed, but despite the lower overall incidence of copulations their relative mating success increased (Table 1). Furthermore, recorded MR of F males were significantly higher than those of D males at both 2.5 and 5 h of desiccation (Fig. 1), whereas relative male mating success showed a significant change between these two stress levels (Table 1). In addition, significant differences in mating success under nonstressful conditions could not be explained by the similar recorded MR for D and F males (Fig. 1), and therefore we conclude that differences in mating success did not simply reflect differences in activity between F and D males.

Adaptive divergence has been shown to be associated with sexual isolation in laboratory populations of both *D. melanogaster* and *D. pseudoobscura* (Kilias et al. 1980; Dodd 1989; Dolgin et al. 2006). *D. melanogaster* populations adapted to different temperature and humidity environments still exhibited nonrandom mating preference after 31 generations in a common environment, highlighting the genetic basis for the observed reproductive isolation (Kilias et al. 1980). In this study, we used a range of desiccation stress levels analogous to the increasing desiccation stress as resources become sparse in areas that provide an ecological barrier for species distribution. We show that selection for desiccation resistance in *D. melanogaster* results in significant deviations from random mating. Adaptation confers fitness advantages, including higher male mating success in comparison with nonadapted flies under stressful conditions. Higher mating success of adapted genotypes in stressful environments could interrupt gene flow between ancestral and locally adapted populations, thus triggering an evolutionary divergence of peripheral stress-resistant populations (Garcia-Ramos and Kirkpatrick 1997; Lenormand 2002). Therefore, we suggest that the observed higher mating success of D males under stressful conditions, together with their compromised mating performance under milder conditions, may indicate a possible mechanism for divergence of stress-resistant populations in nature.

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