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Robert A. Krebs
Cleveland State University, r.krebs@csuohio.edu

Kimberly A. Thompson
Cleveland State University

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A genetic analysis of variation for the ability to fly after exposure to thermal stress in *Drosophila mojavensis*

Robert A. Krebs*, Kimberly A. Thompson

Department of Biological, Geological and Environmental Sciences, Cleveland State University, 2121 Euclid Ave., Cleveland, OH 44115, USA

1. Introduction

Most studies of responses to thermal extremes predominantly measure survival (Hoffmann et al., 2003), but the variation measured in response to stress will change with the trait in question, both at phenotypic and genetic levels (Feder, 1996; Hoffmann et al., 1997; Shine et al., 2000). While survival is critical to an organism, physiological stress is predicted to impact fitness before life is threatened (Bennett, 1987a, b). Impacts on traits like locomotion, flight or mating success may be more important to evolutionary change within a species than are effects on survival. As a test of the potential for evolutionary change, we ask how variable are populations of *Drosophila mojavensis* for tolerance to extremes of heat as measured by the ability to fly after exposure to a high, but non-lethal temperature (38 °C for 1 h). This species inhabits thermally diverse environments across southwestern North America, where air temperatures can exceed 38 °C, even in winter (Gibbs et al., 2003).

Populations of *D. mojavensis* vary in many traits across their range. Early research quantified diversity in allozymes (Zouros, 1973; Markow et al., 2002), and four populations studied previously in our laboratory varied in survival, mating behaviors and flight after an exposure to thermal stress (Fasolo and Krebs, 2004). This latter trait is of particular interest to *D. mojavensis* first, because it inhabits the ephemeral habitat of

*Corresponding author. Fax: +1 216 687 6972.
E-mail address: r.krebs@csuohio.edu (R.A. Krebs).

necrotic cactus and must move between 'rots' to reproduce (Gibbs et al., 2003), and second, because the flight musculature is known to be physiologically and biochemically different from other tissues (Gronenberg and Strausfeld, 1990; Scaraffia et al., 1997; Patton and Krebs, 2001).

To assess genetic variation in the tolerance of *Drosophila* flight muscle to heat stress, we studied the ability for young, but mature adults of *D. mojavensis* to fly following an exposure to thermal stress. Four specific populations were chosen as they link present results to previous work, and by using these strains isolated from geographically separate populations of the species, patterns of genetic inheritance of the variation are more likely to be identified. Our approach was modeled from the analysis of pupation behavior by Bauer and Sokolowski (1988), where we initially conducted all pair-wise reciprocal F_1 crosses among the four strains. Those crosses were followed up by tests on backcrosses, choosing two of the possible pairs after assessing the initial results on strain differences. In sum, the strongest effects were for significantly increased performance by strain hybrids relative to their parental types. Despite a potential for mitochondrial variation to have a big impact on flight performance, no maternal effects were identified, and the contribution of the X chromosome was small.

2. Material and methods

Three of the *D. mojavensis* strains used in these comparisons were collected in 1999 by Dr. Teri Markow at the University of Arizona: Santa Catalina Island (CI), 43 km west of Los Angeles, the one site where this species uses prickly pear cactus (*Opuntia*) as a substrate, Ensenada de los Muertos (EN), 35 km NE of La Paz, along the Cape Region of Baja California, Mexico where agria cactus (*Stenocereus gummosus*) is the most common host plant, and Santa Rosa Mountains (SR) in southern Arizona, USA, a site dominated by organ pipe cactus (see Heed and Mangan, 1986; Markow et al., 2002; Fasolo and Krebs, 2004, for information on these sites). We collected the fourth population in San Carlos (SC), Mexico, in January 2000 (Krebs et al., 2000), where organ pipe also is the most abundant host plant.

All *D. mojavensis* strains had been maintained on a standard cornmeal–yeast–molasses–agar medium containing tegosept and proprionic acid, where they were reared in a 25 °C incubator. Several sets of experiments indicate that stress tolerance remains fairly constant in laboratory populations of *Drosophila*, at least for several years (Krebs and Loeschcke, 1999; Krebs et al., 2001). Furthermore, we retested tolerance as measured by survival in all four strains (Table 1), verifying consistency for patterns of tolerance observed earlier (Fasolo and Krebs, 2004).

The genetic crosses were set up in early 2004. In Part I, virgin males and females of each strain were collected and used either to start pure strain cultures or reciprocal F_1 crosses between these lines, giving 16 sets of crosses: four within each strain, and two reciprocal sets of each of the six possible crosses between strains. Two bottles of each cross were prepared, and these adults were transferred to fresh media after three and six days to ensure sufficient progeny for analysis. As new flies emerged, they were collected within 24 h, and 10–12 males or females were placed in each glass vial. All experiments examined 7-day-old flies. Approximately 10 replicates each for males and for females from each cross were collected over an 8-day period.

In Part II, reciprocal cross-progeny and flies from the parental strains were collected for two population pairs to create all possible backcross progeny. The two pairs were Catalina Island, which was the most heat tolerant strain, and it was crossed either with Ensenada de los Muertos, the population from the tip of the Baja peninsula, or with Santa Rosa, the population from southern Arizona. As there are four reciprocal F_1 progeny types possible, eight different backcrosses were made for each pair of strains. Again, 10 replicate groups of males and females were collected for each cross, with treatment conditions identical throughout all experiments.

2.1. Tests of flight after stress

To obtain flies for experiments, all rearing bottles were cleared daily (adult flies removed and discarded)

Table 1
Survival and flight performance of four *D. mojavensis* populations pooled for males and females and across all experiments

Strain	Survival to 41 °C	Flight to 38 °C (Exp. 1)
Catalina Island (CI)	0.882 ± 0.046 A	0.555 ± 0.037 A
San Carlos (SC)	0.755 ± 0.068 AB	0.277 ± 0.081 B
Santa Rosa (SR)	0.556 ± 0.066 BC	0.450 ± 0.050 AB
Ensenada de los Muertos (EN)	0.061 ± 0.029 C	0.346 ± 0.051 B

Groups marked with the same letter do not differ statistically based on Tukey's multiple comparisons

prior to collection to ensure that collected flies were virgins. For collection, all flies were anesthetized with CO_2 gas and separated by sex under a dissection microscope. Approximately 10–12 flies were then placed into fresh glass holding vials containing roughly 2 ml of medium and a sprinkling of dry yeast. Once separated, *D. mojavensis* were held for 7–9 days before use to allow the flies to reach sexual maturity (Markow, 1982). Flies were transferred into fresh vials midway through the holding period to prevent bacterial growth and to promote maximum health.

High temperature stress was applied by immersing glass vials holding 7-day-old *D. mojavensis* adults for 1 h in water baths (Polyscience) heated to 38.0 °C. The baths were monitored with two physiological mercury thermometers from which we verified that water temperature did not vary more than 0.1 °C during experiments. Each vial of flies contained an agar-based medium; they were stuffed with a damp cotton ball, and then sealed with a rubber stopper before they were placed inverted within the baths. After exposure, the vials were lifted from the water and the stoppers were removed. After 1 h, flight was scored based on the ability to take off and fly a distance of 10 cm or more. If a fly would not take off, not fly when probed with a camel hair paintbrush, or would repeatedly land less than 10 cm away, it was scored as no flight.

2.2. Statistical analysis

Data, which were recorded as frequencies within each vial, were arcsine-square root transformed to increase the variance in the extremes of the distributions. A model III, three-way fixed-factor GLM was run in SAS for male parent origin, female parent origin, and gender (SAS Institute, 1998). The design was balanced, but the GLM procedure was used due to a few missing data. While complete analyses on all data tested for large effects within experiments such as those that may occur with heterozygote inferiority or superiority, variation based on parent origin was not random. Therefore, reduced GLM analyses and *t*-tests were also applied for specific contrasts, for example: tests of maternal effects, X chromosome contribution, and the co-dominant/additive nature of variation.

3. Results

3.1. Flight in population hybrids

The four strains varied much less for ability to fly after heat stress than they did for survival to extreme heat (Table 1). More variation was apparent by comparing the F_1 hybrid progeny derived among these strains; all flew at a significantly greater frequency than did flies from their parent strains (Fig. 1). In the analyses of variance, the interaction effect between the origin of the male parent and female parent was highly significant in all six crosses (Table 2; $P<0.001$), and it often explained more than 40% of the total variance. No other interaction effects were significant, and tests of the main effects, male parent and female parent, were largely confounded by this huge interaction effect. Females flew at a higher frequency than did males across all groups (GLM, pooling all comparisons, $P<0.01$), but differences were not large enough to show general significance in the subsets of data (Table 2).

3.2. Flight in backcross progeny

Backcross tests where all F_1 hybrid progeny were crossed with their parent strains created 16 separate hybrid groups per pair of strains (male and female progeny of each back-cross paired to males and females of each parental strain). Therefore, these tests were restricted to progeny between CI and SR and between CI and EN. For them, several specific contrasts are relevant to explore differences between strains rather than ANOVA for all groups combined. For the cross between CI and EN, a higher proportion of CI genes increased flight frequency: flies with only EN genes flew at a mean rate of 0.54 ± 0.06; 25% CI added to EN flew at 0.67 ± 0.03; 50% CI/EN (Exp. I) at 0.74 ± 0.03, and 75% CI/EN flew at 0.79 ± 0.02. With hybrid vigor, a slowing of the benefit of CI is expected as 100% CI flew at a rate of only 0.64 ± 0.06, a result consistent with the reciprocal cross results.

Effects of an X chromosome contribution to variation were tested by comparing female progeny in crosses 14–16 and crosses 18–20 (Table 3A); here differences were small and not significant. No general trend suggested any effect of the X chromosome in male progeny either, nor did pairs of crosses that differed only in cytoplasm (Table 3A, rows 5 and 6 versus 7 and 8 or 9 and 10 versus 11 and 12) suggest any maternal effects in the CI or EN lines.

Where SR was used instead of EN for backcrosses from the F_1 hybrids, CI genes again aided flight, but only slightly (Table 3B). Flies that possessed all SR genes flew at a mean of 0.52 ± 0.08; those with a genome composed 25% from CI and 75% from SR flew at a frequency of 0.55 ± 0.03; a 50% CI/SR mix (Exp. I) flew at 0.64 ± 0.05, and 75% CI, 25% SR flew at 0.62 ± 0.03. All groups with mostly CI genomes were similar to the 100% CI flies, which flew at 0.64 in this second experiment, and the effect of hybrid increase was less apparent. An X chromosome effect is only suggestive based on a contrasts between males in one set of comparisons (Table 3B): rows 9 and 11 where half of the males have the SR X chromosome and half the CI X chromosome (0.50 ± 0.09 and 0.49 ± 0.08) versus 17 and

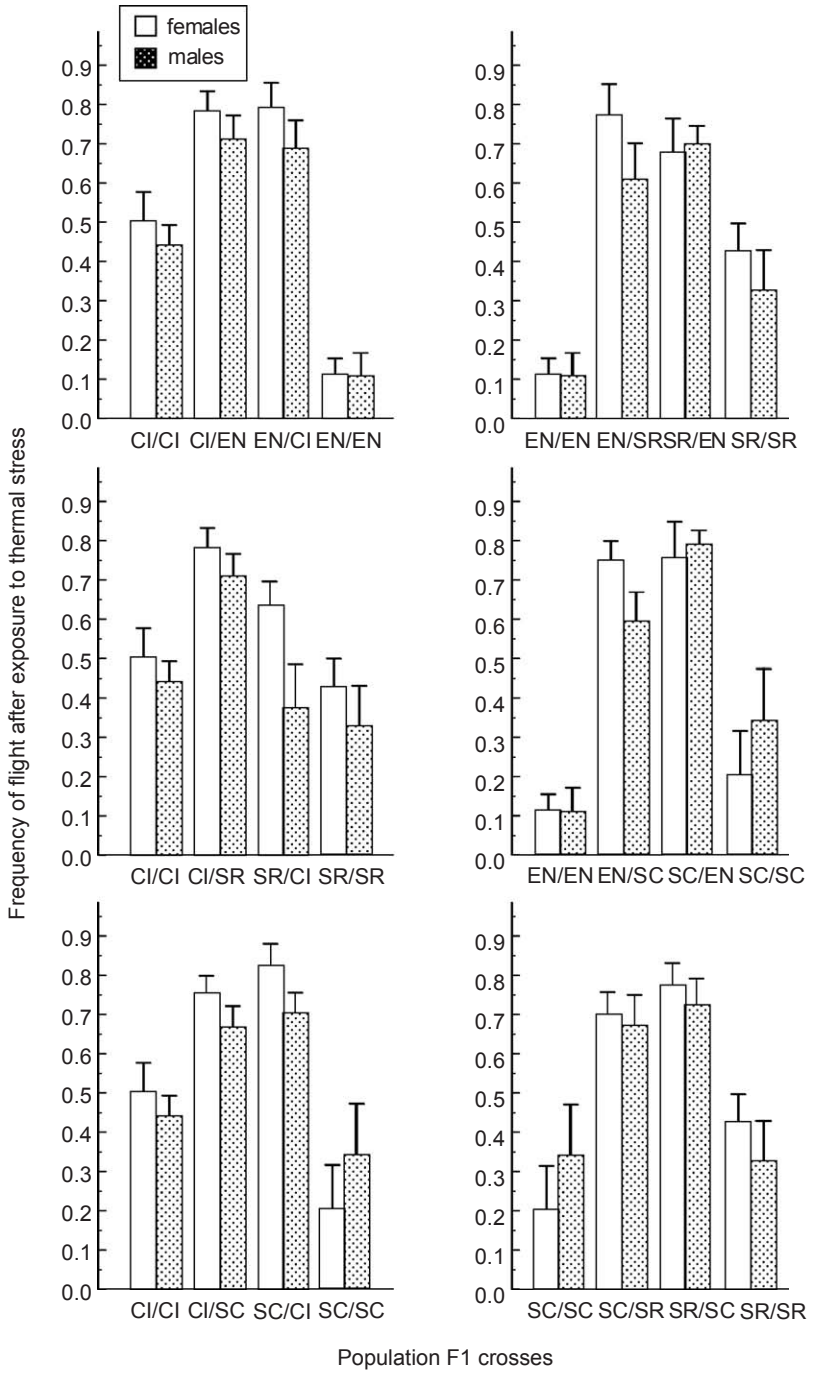

Fig. 1. The frequency of flight in females (clear) and males (speckled) from four *D. mojavensis* populations and their F_1 hybrid progeny after exposure to thermal stress. Each cross is indicated as female parent/male parent, and results for pure-population flies are repeated within the figure for clarity in presentation.

19 where all males have the CI X chromosome (0.60 ± 0.08 and 0.58 ± 0.09). In females, similar contrasts can be made from rows 14 and 16, where half the females are heterozygous for the X and half are homozygous for the SR X chromosome (0.62 ± 0.06 and 0.57 ± 0.08), and crosses 18–20 where females are either homozygous for CI or are heterozygous (0.58 ± 0.10 and 0.68 ± 0.06); here no effect was observed.

Table 2
Significance of General Linear Models for all six comparisons of F_1 hybrids between *D. mojavensis* strains

Source	CI vs. SR	CI vs. SC	CI vs. EN	SR vs. SC	SR vs. EN	SC vs. EN
Sex	*	NS	NS	NS	NS	NS
Male parent	NS	*	***	NS	*	*
Female parent	***	NS	***	NS	**	NS
Sex * male par	NS	NS	NS	NS	NS	NS
Sex * fem par	NS	NS	NS	NS	NS	NS
Male par * fem par	***	***	***	***	***	***
Sex * mal * fem	NS	NS	NS	NS	NS	NS

NS: not significant.
*$P<0.05$.
**$P<0.01$.
***$P<0.001$.

3.3. Test of cytoplasmic effects

Contrasts comparing the source of maternal cytoplasm also can be made from Table 3 (rows 6 and 8; 10 and 12). Within the first set of crosses, daughters of reciprocal crosses flew at a higher frequency if they possessed the CI cytoplasm rather than the SR cytoplasm (T-test, $P<0.05$), although the same contrast using CI and EN showed no significant difference based on cytoplasm. Differences in the backcross progeny experiments refuted any significance to the effect of cytoplasm. Females with an SR cytoplasm flew at a higher frequency than those with a CI cytoplasm, indicating that variation among groups differing in cytoplasm was not consistent (Table 3, row 6 versus 8 and row 10 versus 12).

4. Discussion

The genes that underlie variation in flight following exposure to thermal stress are probably very different from factors affecting variation in other traits measured under stress. Populations that have a high tolerance for heat as measured by a trait like survival may be one of the lower performing populations for flight (Fasolo and Krebs, 2004). Hoffmann et al. (1997) first highlighted how tests of stress tolerance give different patterns of relative tolerance among strains of *D. melanogaster* when the trait under study is changed. Their results were supported by genetic tests for correlation across several developmental and life history traits after stress exposures in *D. buzzatii* (Krebs and Loeschcke, 1995, 1999), a close relative of *D. mojavensis* in the mulleri subgroup of *Drosophila* (Patterson and Stone, 1952).

Despite an expectation that numerous biochemical pathways affect thermal adaptation (Feder, 1996), few studies quantify the number of traits responsible for variation among populations (Hoffmann et al., 2003). Here, hybrid offspring between all pairs of strains flew much more frequently than did parental flies indicating that at least four different genetic systems help adult *D. mojavensis* maintain flight in a hot environment. These effects are predominantly autosomal as males and females were similarly affected. The origin of the X chromosome and the cytoplasm provided no consistent differences of significant magnitude under the sample sizes examined here. The backcross studies supported this multigenic model for variation. Increasing chromosome proportion from the CI line increased tolerance both in comparison to EN and to SR, and hybrids continued to fly at much greater frequencies than did flies from either parental strain.

Therefore, a model of simple hybrid vigor, for example, assuming that each of the laboratory lines were inbred, seems insufficient to explain the results. Inbreeding has been shown to affect survival tolerance to heat in *D. suboscura* (Bowler and Hollingsworth, 1965) and in *D. melanogaster* (Dahlgaard et al., 1995), but not to knockdown from heat (Dahlgaard and Hoffmann, 2000). Furthermore, tests for survival after heat stress using two of these same strains did not produce any hybrid superiority, and instead inheritance followed a dominant/recessive pattern (Fasolo and Krebs, 2004). For these reasons, and the consistency of strain differences over time when measured by survival after heat stress, the hybrid benefit appears specific to the flight phenotype and not a consequence of inbreeding depression.

Past work using *Drosophila* to study tolerance variation in survival also suggests that inheritance of stress tolerance changes with the trait studied (Hoffmann et al., 1997). In *D. melanogaster*, one or more important genes for survival after heat stress occur on the X chromosome (Krebs et al., 1996), while in *D. buzzatii* an unusual element that functions only to improve survival tolerance of males occurred in one strain (Krebs and Loeschcke, 1996). In contrast to survival, knockdown may show a more multigenic effect (Norry et al., 2004), although Gilchrist and Huey (1999)

Table 3
Frequency of flight for (A) reciprocal cross Catalina Island (CI) and Ensenada de los Muertos (EN) hybrid progeny backcrossed to the parent strains and (B) flight frequency for the backcrosses between CI and the Santa Rosa, AZ strain (SR)

	Male parent	Female parent	Gender	Mean frequency	Standard error
Part A					
1	CI	CI	Males	0.568	0.077
2	CI	CI	Females	0.705	0.091
3	EN	EN	Males	0.505	0.080
4	EN	EN	Females	0.571	0.103
5	EN	ENM CIF	Males	0.690	0.092
6	EN	ENM CIF	Females	0.568	0.093
7	EN	CIM ENF	Males	0.638	0.056
8	EN	CIM ENF	Females	0.675	0.071
9	CI	ENM CIF	Males	0.727	0.082
10	CI	ENM CIF	Females	0.854	0.072
11	CI	CIM ENF	Males	0.722	0.067
12	CI	CIM ENF	Females	0.854	0.048
13	ENM CIF	EN	Males	0.781	0.044
14	ENM CIF	EN	Females	0.755	0.068
15	CIM ENF	EN	Males	0.673	0.069
16	CIM ENF	EN	Females	0.714	0.081
17	ENM CIF	CI	Males	0.684	0.055
18	ENM CIF	CI	Females	0.863	0.048
19	CIM ENF	CI	Males	0.763	0.076
20	CIM ENF	CI	Females	0.872	0.032
Part B					
1	CI	CI	Males	0.568	0.073
2	CI	CI	Females	0.705	0.087
3	SR	SR	Males	0.487	0.123
4	SR	SR	Females	0.548	0.105
5	SR	SRM CIF	Males	0.550	0.072
6	SR	SRM CIF	Females	0.525	0.102
7	SR	CIM SRF	Males	0.506	0.107
8	SR	CIM SRF	Females	0.582	0.092
9	CI	SRM CIF	Males	0.502	0.094
10	CI	SRM CIF	Females	0.738	0.044
11	CI	CIM SRF	Males	0.492	0.084
12	CI	CIM SRF	Females	0.787	0.064
13	SRM CIF	SR	Males	0.563	0.067
14	SRM CIF	SR	Females	0.622	0.061
15	CIM SRF	SR	Males	0.496	0.109
16	CIM SRF	SR	Females	0.571	0.084
17	SRM CIF	CI	Males	0.619	0.075
18	SRM CIF	CI	Females	0.576	0.100
19	CIM SRF	CI	Males	0.583	0.090
20	CIM SRF	CI	Females	0.678	0.064

suggested only a couple of genetic differences could be sufficient to explain variation in their lines. These effects could be more relevant to fitness than survival, as the knockdown temperature used in these studies for *D. melanogaster* was 36.5–37 °C, comparable temperatures to the 38 °C used here to knock out flight in *D. mojavensis*. Another possible link between heat resistance assayed by flight (based on our unpublished data) and knockdown is a similar tendency for conditioning treatments to provide little protection to the stress (Sørensen et al., 2001). Clearly, high-temperature stress affects an organism in many ways, and the variation underlying this tolerance should depend upon how tolerance to stress is measured. Variation can change with the stress level and the tissue(s) important to the trait under study.

For these reasons, specific candidate traits have been examined extensively for their role in thermotolerance. One focus has been on molecular chaperones, especially Hsp70, which is critical for survival in natural populations of *D. melanogaster* (Krebs and Feder, 1997a). In *D. mojavensis* adults, Hsp70 is induced at 38 °C, and peaks at 39 °C (Krebs, 1999), but expression in the thorax is less than half that in either the head or abdomen (Patton and Krebs, 2001). Flesh flies likewise vary among tissues in heat-induced expression of Hsp72, which is not present in flight muscle (Denlinger et al., 1991), possibly to avoid effects that Hsp70 class proteins have on metabolism and growth when present at high concentrations (Krebs and Feder, 1997b, 1998). However Hsp70 is only one of a suite of changes that make up what is known as the heat-shock response (Feder and Hofmann, 1999), and other stress responses may protect flight musculature (ElWadawi and Bowler, 1995). Oxidative respiration in mitochondria of flies, for one, can be protected by a conditioning treatment prior to exposure to a thermal stress (ElWadawi and Bowler, 1996).

Other candidate systems have recently been shown to influence thermotolerance, often in a tissue-specific manner. Tulin and Spradling (2003) report an association of PARP-1 with chromosomal "puffs" around the heat shock genes after exposure to stress. PARP is critical to transcriptional activation throughout development in *Drosophila* (Kraus and Lis, 2003; Tulin et al., 2002). Interestingly, in mammals, PARP-1 may actually attenuate inducible Hsp70 expression (Zingarelli et al., 2004), and McLaughlin et al. (2003; see also McLaughlin, 2004) further report that PARP and other agents that block conditioning effects on stress concurrently block Hsp70 upregulation. Those findings are particularly relevant to the flight phenotype of our flies because a preliminary study suggested that exposure of *D. mojavensis* to 36 °C for 1 h prior to exposure to the higher stress increased activity (as running across the table), but failed to ameliorate consequences to flight.

Another possible candidate system is calcium release. Increasing extracellular calcium concentrations enabled muscle fibers in larval *Drosophila* to remain active at temperatures up to 5 °C higher than without the calcium, an effect that mirrored benefits from physiologically conditioning the flies to heat prior to testing (Barclay and Robertson, 2003). This response to heat

could be either an effect on residual calcium levels or on the presynaptic targets of calcium, as also suggested in the locust-leg model for heat–stress responses in muscle tissue (Barclay and Robertson, 2001).

Overall, many physiological changes occur when an organism encounters high temperatures. Several possibilities can act similarly or in groups to reduce performance in traits like flight. Determining what characteristic fails first in one fly or fly strain may never be simple, but one answer stands out; there is no one answer. Each strain may differ in the protein or physiological system most susceptible to stress.

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