

Seasonal stress drives predictable changes in inbreeding depression in field-tested captive populations of *Drosophila melanogaster*

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Recent meta-analyses conducted across a broad range of taxa have demonstrated a strong linear relationship between the change in magnitude of inbreeding depression under stress and stress level, measured as fitness loss in outbred individuals. This suggests that a general underlying response may link stress and inbreeding depression. However, this relationship is based primarily on laboratory data, and it is unknown whether natural environments with multiple stressors and fluctuating stress levels alter how stress affects inbreeding depression. To test whether the same pattern persists in the field, we investigated the effect of seasonal variation on stress level and inbreeding depression in a 3-year field study measuring the productivity of captive populations of inbred and outbred *Drosophila melanogaster*. We found cold winter temperatures were most stressful and induced the greatest inbreeding depression. Furthermore, these data, collected under natural field conditions, conformed to the same predictive linear relationship seen in *Drosophila* laboratory studies, with inbreeding depression increasing by 0.17 lethal equivalents for every 10 per cent increase in stress level. Our results suggest that under natural conditions stress level is a primary determinant of the magnitude of inbreeding depression and should be considered when assessing extinction vulnerability in small populations.

Keywords: inbreeding depression; environmental stress; stress level; natural conditions; seasonal variation

1. INTRODUCTION

Environmental threats such as climate change, destruction or loss of habitat and pollution can impose immense fitness costs that jeopardize the survival and persistence of natural populations [1–4]. These threats define forms of environmental stress and, because stress is commonly thought to magnify the deleterious fitness consequences that result from inbreeding [5,6], they are likely to render small populations especially vulnerable to such adverse conditions [2,4,7,8]. Stressors, such as extreme temperatures, pathogens and exposure to chemicals, are predicted to amplify the fitness costs associated with inbreeding (i.e. inbreeding depression) by altering the expression of deleterious alleles (genetic load) in homozygous individuals [5,9], which in turn can heighten extinction risk for small populations [8,10]. However, although a recent meta-analysis found average inbreeding depression is elevated under stressful conditions [9], less than half (48%) of the 53 cases reviewed showed a statistically significant increase, and 24 per cent of cases actually report a decrease in inbreeding depression under stress, a pattern that has fuelled continued controversy over the interaction between inbreeding and stress [9,11,12]. This controversy has largely been resolved by including a measure of the magnitude of stress in the analysis

[5,13]. This addition has led to the finding of a strong linear relationship that applies across a diverse array of plant and animal species between the magnitude of effect a stress has on the fitness of outbred individuals (stress level) and inbreeding depression (measured as lethal equivalents [13]; i.e. the slope of the relationship between the natural log of fitness and the level of inbreeding).

The finding of such a general relationship points to the possibility of a very general underlying phenomenon. However, the majority of research included in these reviews has been conducted under controlled laboratory conditions using artificial stressors that may not be representative of complex natural environments where exposure to multiple abiotic and biotic stresses could significantly alter inbreeding–environment interactions owing to synergistic effects of combined stresses on survival [14,15]. In addition, laboratory studies have imposed stress levels ranging from zero stress up to a greater than 80 per cent reduction in fitness [5], but it is unknown what part of this range is representative of stress levels populations experience in the wild or to what extent stress levels vary over time (e.g. through seasonal fluctuations). As a result, it is unclear how natural variation in stress levels will impact the relationship between inbreeding depression and exposure to stressful conditions in natural populations.

The dynamics of inbreeding–stress interactions in natural populations are poorly understood [8,16], despite implications for adaptation to anthropogenically induced

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environmental changes [1,4,9], purging of genetic load [17] and the survival of small endangered populations [4,8,10]. Among the studies reviewed by Armbruster & Reed [9] only 9 of 34 measured inbreeding depression under field conditions and only 4 measured natural temporal variation in levels of inbreeding depression in wild populations [18–21]. For example, in populations of Darwin's finches inbreeding depression for adult and juvenile survival was found to be strongly influenced by food availability and the presence of competitors [19], while in song sparrows periods of rain were shown to significantly increase inbreeding depression for hatching success [20]. However, none of the studies were able to quantify the level of stress. To determine whether natural stress is qualitatively and quantitatively similar to laboratory stress, we need to determine whether the linear relationship found between stress level and inbreeding depression in laboratory studies holds when populations are challenged by naturally complex and unpredictably stressful environments.

We investigated this relationship, focusing on the effect of seasonal stress. We established a 3-year semi-natural field study in Riverside, California to measure these fluctuations in stress level, and to determine whether these fluctuations induced the expected changes in inbreeding depression. Our study focused on the unpredictable stresses imposed by seasonality, using as our measure of fitness the productivity of small one-generation bottle populations of *Drosophila*. It is well established that both extreme high and low temperatures can drastically reduce viability in *D. melanogaster*; however, it is unknown how natural seasonal fluctuations might challenge the physiological limits of this species, therefore affecting both stress levels and inbreeding depression. We found that cold winter conditions were the most stressful for population productivity. We also showed that a strong positive linear relationship exists between increasing stress levels and the magnitude of inbreeding depression expressed in populations exposed to natural seasonal variation.

2. MATERIAL AND METHODS

(a) Measurement of productivity of bottle populations in the field and laboratory

Inbred populations were created from a set of lines established approximately 18 months earlier following at least five generations of full-sib mating and then by imposing a further 20 generations of full-sib mating just prior to our experiments. These lines were started from the offspring of single females collected in the University of California, Riverside campus orange grove, and maintained as bottle populations between the two periods of inbreeding. According to theoretical expectation more than 25 generations of full-sib mating yields inbred lines that are close to completely homozygous ($F > 0.99$) [22]. However, recent work [23] suggests the F level will actually be slightly less ($F = 0.95–0.99$). During the inbreeding process lethal and some other highly deleterious alleles expressed under benign laboratory conditions would have been purged; however, such large-effect alleles are rare (given mutation–selection balance). By contrast, alleles with smaller deleterious effects will have been randomly fixed in proportion to their frequency. This fixation, combined with the environmental dependence of many deleterious alleles, and thus inbreeding depression [4,16], means that these

highly inbred lines are expected to effectively sample the vast majority of the genetic load found in wild populations.

Outbred populations were created by combining an equal number of individuals from each of 10 inbred lines. These populations were maintained in the laboratory (25°C) for four generations prior to use in experiments. Between field seasons, the inbred lines were maintained in the laboratory (25°C), and prior to the start of each field season three separate outbred lines were recreated, always using the same 10 inbred lines. Six of these inbred lines were randomly chosen and used in all field and laboratory experiments.

To initiate an experimental set, we created three experimental bottle populations of 30 males and 30 females (1 to 3 days old) from each of the six inbred and the three fourth-generation outbred stock populations. Each set of 27 bottles used in field testing was placed in full shade under a single tree in the orange groves located on the campus of the University of California, Riverside. Field tests were performed during the three hottest (July, August and September) and three coldest (December, January, and February or March) months. Temperature and humidity were recorded hourly from the time the populations were placed in the field to the time the last progeny were collected using temperature sensors (Dallas Maxim iButton data loggers) placed on the ground next to the experimental bottles. Preliminary experiments indicated that temperatures on the ground directly next to experimental bottles were not different from inside bottles (average difference = $\pm 1^\circ\text{C}$; data not shown). In addition, during the first month of each season, an additional set of these bottle populations was placed simultaneously in a laboratory incubator at a constant temperature of 25°C.

Bottles placed in the field contained 50 ml of standard cornmeal molasses media and were covered with four layers of cheesecloth to allow airflow. A custom-made device made from a small aluminium rod was fitted to the opening of the bottles and served to prevent food (which sometimes detached when the bottle was inverted) from trapping or killing emerged flies when they were collected. Each bottle was placed in a separate plastic beaker that was secured to the ground with a nail and covered with a ring of Tanglefoot ant/insect deterrent. A metal cage was placed over all the bottles to prevent tampering by raccoons and other small mammals. Despite these precautions, across the three years, 24 of the 135 outbred and 36 of the 270 inbred field bottle populations were lost. Bottles placed under laboratory conditions contained the same amount and type of media, but had foam plugs and no aluminium device inside.

The population productivity of each bottle population was measured as the total number of adult progeny produced in a single generation. Population productivity is a product of parental fecundity and fertility, and of offspring survivorship. As such, it represents a broad measure of fitness [24,25] and is a useful indicator of short-term population persistence. Adults were left in the bottles until the first pupae were observed in any bottle of a set. Eclosing offspring were collected and removed every day for the first 5 days and then once every 3–4 days until there were dark pupae observed in the 'timing control' bottles, which served as a criterion for determining the end of first generation and beginning of second generation. For each set, a timing control bottle was initiated when the first pupae were observed. This timing bottle, containing 60 outbred flies on fresh food, provided a conservatively short estimate of the time it was taking for a second generation to develop in the experimental bottles. When dark (immediately pre-eclosion) pupae were

observed in the timing control bottle, this marked the end of offspring collection from the experimental bottles. Note that this measure of generation time was defined by larval development time rather than absolute time, thus controlling for the changes in metabolic activity resulting from changes in temperature.

(b) *Stress level*

Stress level was measured as the proportion reduction in outbred population productivity under field conditions relative to benign laboratory conditions, similar to a measure used previously [5]:

$$\text{stress level} = 1 - \left(\frac{\text{productivity}_{\text{outbred field}}}{\text{productivity}_{\text{outbred lab}}} \right).$$

By definition, a zero level of stress will result in equivalent inbreeding depression under benign and (supposedly) stressful conditions [5]. Outbred productivity under benign laboratory conditions did not vary significantly across the different years or seasons of this study (see §3), therefore in order to calculate stress level separately for each month of summer and winter across the 3 years of the study, we used the same estimate of average outbred productivity under laboratory conditions for calculating stress levels in the field across all months within a given season of a year. For example,

$$\text{stress level}_{\text{Aug year 1}} = 1 - \left(\frac{\text{outbred productivity}_{\text{field Aug year 1}}}{\text{outbred productivity}_{\text{lab summer year 1}}} \right).$$

(c) *Lethal equivalents*

For each inbred line, we calculated the number of lethal equivalents (β) under benign laboratory conditions and stressful field conditions (β_{benign} and β_{stress} , respectively). The number of lethal equivalents is a measure commonly used to compare the effects of inbreeding on fitness across studies, species/taxa and environments [9,11]. Lethal equivalents were calculated according to the following equation [26]:

$$\beta = \frac{-\ln(w_i/w_o)}{F},$$

where w_i and w_o are the mean fitness (in this case, productivity) of inbred and outbred populations, respectively, and F is the level of inbreeding. Our inbred populations are highly inbred and considered to be completely homozygous ($F \approx 1$; see earlier text). We calculated the number of lethal equivalents for each individual inbred line for each month of summer and winter, as well as laboratory conditions. Each individual set of bottle populations placed either into the field or in the laboratory incubator consisted of three outbred lines and six inbred lines, therefore we used the average outbred population productivity (w_o) per set to calculate lethal equivalents for each of the inbred lines in the same set. For field conditions, this resulted in an estimate of lethal equivalents for each month of winter and summer, while lethal equivalents were estimated once per season under laboratory conditions.

(d) *Data analyses*

We first analysed productivity differences among the outbred populations in the field, to determine differences across YEAR (year 1, 2, 3), SEASON (summer, winter) and MONTH (nested within SEASON and YEAR), recalling that each outbred population was replicated three times, using ANOVA (SAS v. 9.1). YEAR was defined across calendar years to contain one winter and one summer, with winter comprising December, January and February/March, and

summer comprising July, August and September. We also analysed productivity differences among the outbred populations under benign laboratory using ANOVA that included YEAR and SEASON.

To convert productivity to stress level, the seasonal productivity values of the outbred populations were corrected by the laboratory productivity for that season in that year. We compared stress levels experienced during the winter and summer seasons (SEASON) and across years (YEAR: 1–3, each consisting of one summer and one winter season). Given the consistent dominance of seasonal effects relative to monthly variation (see §3), replicates were pooled across the three outbred lines within treatment month and each month was then treated as a replicate within season.

Inbreeding depression, measured as the number of lethal equivalents, was analysed using ANOVA with the main effects ENV (laboratory, field summer, field winter), YEAR and LINE (six inbred lines). ENV and YEAR were fixed effects while LINE was random. Similar to the above analysis of stress levels, each monthly estimate of inbreeding depression for the six inbred lines was treated as a replicate within season. Interactions with a p -value greater than 0.25 were removed, and the analysis was re-run [27]. Post hoc multiple comparisons were calculated using a Tukey's test to determine whether inbreeding depression varied significantly across the field (winter and summer) and benign (laboratory) environments.

To test the hypothesis that the difference between inbreeding depression under stressful field and benign laboratory conditions ($\Delta\beta = \beta_{\text{stress}} - \beta_{\text{benign}}$) is greatest during the most stressful months of the year, a model I ordinary least-squares linear regression (both unconstrained and constrained to pass through the origin) was performed comparing $\Delta\beta$ and the level of stress for each month of the study. The sample variance on the y -axis ($\Delta\beta$) was found to be $4.5\times$ larger than that on the x -axis (stress level), which is consistent with our expectation that the error variance of $\Delta\beta$ is much greater than that of stress level, and allows us to use a model I rather than model II regression [28]. We also tested for nonlinear (quadratic and cubic) relationships and compared model fit to the simple first-order linear regression using an F -test. In addition, it should be noted that the data points within a season in a given year are not independent because the same laboratory data are used to define β_{benign} . We therefore performed two additional regressions based on truly independent data, comparing $\Delta\beta$ and the level of stress for each season within each year (six data points), and using a single constant laboratory value of productivity to calculate β_{benign} and stress level across all months and seasons (15 data points).

Following Fox & Reed [5], we performed simulations to measure the magnitude of the inherent correlation between the variables stress level and $\Delta\beta$. We randomly generated 10 datasets of 10 000 cases where inbred and outbred fitnesses were sampled from a uniform distribution using a random number generator in MATLAB (version for Windows) and then used to calculate stress level and the number of lethal equivalents (β_{stress} and β_{benign}). A linear regression was then run on the difference in lethal equivalents under stressful and benign conditions ($\Delta\beta$) and stress level. On the basis of reported levels of stress and inbreeding depression in *Drosophila* studies, the upper bounds of these measures were set at 75 per cent (stress level) and 50 per cent (inbreeding depression). Two levels of inbreeding ($F = 0.25$ or 1.00) were used in separate simulations (five datasets per F).

Table 1. Seasonal variation in population productivity across 3 years under (a) laboratory and (b) field conditions, and (c) the resulting field stress levels. Productivity = the number of adults produced by a population of 30 females \pm s.e. Stress level = $1 - [\text{OB}_{\text{field}}/\text{OB}_{\text{lab}}]$, i.e. the loss of productivity of outbred populations in the field (OB_{field}) relative to productivity under laboratory conditions (OB_{lab}). The seasonal averages were calculated across relevant sample months \pm s.e.

	year 1		year 2		year 3	
	outbred	inbred	outbred	inbred	outbred	inbred
(a) laboratory productivity						
summer	561 \pm 17	454 \pm 19	556 \pm 12	455 \pm 14	430 \pm 10	297 \pm 15
winter	567 \pm 10	436 \pm 20	486 \pm 18	351 \pm 19	488 \pm 14	347 \pm 24
(b) field productivity						
summer						
July	—	—	535 \pm 19	314 \pm 20	308 \pm 13	237 \pm 17
August	382 \pm 20	297 \pm 21	444 \pm 33	338 \pm 22	510 \pm 18	212 \pm 15
September	499 \pm 19	322 \pm 18	448 \pm 30	297 \pm 14	448 \pm 15	323 \pm 32
winter						
December	—	—	—	—	137 \pm 13	22 \pm 6
January	351 \pm 19	170 \pm 32	285 \pm 10	97 \pm 17	154 \pm 12	32 \pm 8
February/March	298 \pm 8	119 \pm 17	353 \pm 22	128 \pm 20	228 \pm 7	102 \pm 15
(c) stress level						
summer						
July	—	—	0.04	—	0.28	—
August	0.32	—	0.2	—	-0.19	—
September	0.11	—	0.23	—	-0.04	—
yearly average	0.22	—	0.16	—	0.02	—
seasonal average	—	—	0.12 \pm 0.06		—	—
winter						
December	—	—	—	—	0.72	—
January	0.39	—	0.41	—	0.68	—
February/March	0.48	—	0.27	—	0.53	—
yearly average	0.44	—	0.34	—	0.64	—
seasonal average	—	—	0.50 \pm 0.06		—	—

Finally, we compared the relationship between stress level and change in inbreeding depression for the current field study with that for *D. melanogaster* laboratory studies. We identified 22 separate cases from 11 studies using *D. melanogaster* and *D. buzzati* documenting inbreeding depression for larval to adult survival across various types of abiotic and biotic stressors (see the electronic supplementary material, table S1). We selected studies using only larval survival because this fitness component is most closely comparable with population productivity used in the current study. Lethal equivalents used were those published in Armbruster & Reed's [9] review, with the exception of four studies published after 2005 [29–32] and one unpublished study [13]. Electronic supplementary material, table S1 provides information on the studies used in the analysis. We then performed an ANCOVA, with the following factors: STUDY TYPE (current field versus *D. melanogaster* literature) and STRESS LEVEL.

3. RESULTS

(a) Seasonal productivity and stress levels

The overall average productivity (measured as the number of adult offspring produced \pm s.e.) of outbred populations under benign laboratory conditions was 517 ± 5.6 , while in the field it was 440 ± 9.9 in summer and 266 ± 5.8 in winter (table 1a,b). In the laboratory, productivity did not vary across years (YEAR: $F_{2,53} = 3.05$, $p = 0.06$) or seasons (SEASON: $F_{1,53} = 0.41$, $p > 0.25$; SEASON \times YEAR $F_{2,53} = 0.60$, $p > 0.50$). In the field, monthly productivity within season and year varied significantly (MONTH

(SEASON, YEAR): $F_{9,96} = 9.03$, $p < 0.001$); however, despite this within-season variation there was a significant difference between seasons overall (SEASON: $F_{1,9} = 20.18$, $p < 0.002$; i.e. outbred productivity was significantly higher in the summer than winter). This seasonal difference in field productivity was consistent across years (YEAR \times SEASON: $F_{2,9} = 1.92$, $p > 0.20$) and there were no significant differences among years (YEAR: $F_{2,9} = 2.88$, $p > 0.10$).

Because laboratory productivity was consistent over time, variation in the level of stress in the field closely followed variation in field productivity. Thus, contrary to the view that heat stress is extremely detrimental for population survival [30,33], we found that over three seasonal cycles the cold winter months were more stressful for populations of *D. melanogaster* than summer ($F_{1,9} = 20.05$, $p = 0.002$). Winter months were on average four times more stressful than summer months, with outbred populations producing 50 per cent fewer offspring under winter field conditions relative to benign laboratory conditions, but only 12 per cent fewer during the summer (table 1c). These seasonal differences were independent of year (YEAR: $F_{2,9} = 0.48$, $p = 0.633$; SEASON \times YEAR: $F_{2,9} = 3.97$, $p = 0.064$).

(b) The relationship between inbreeding depression and stress level

The cold winter temperatures experienced in the field caused significantly greater inbreeding depression than summer heat (tables 2 and 3; figure 1), matching the

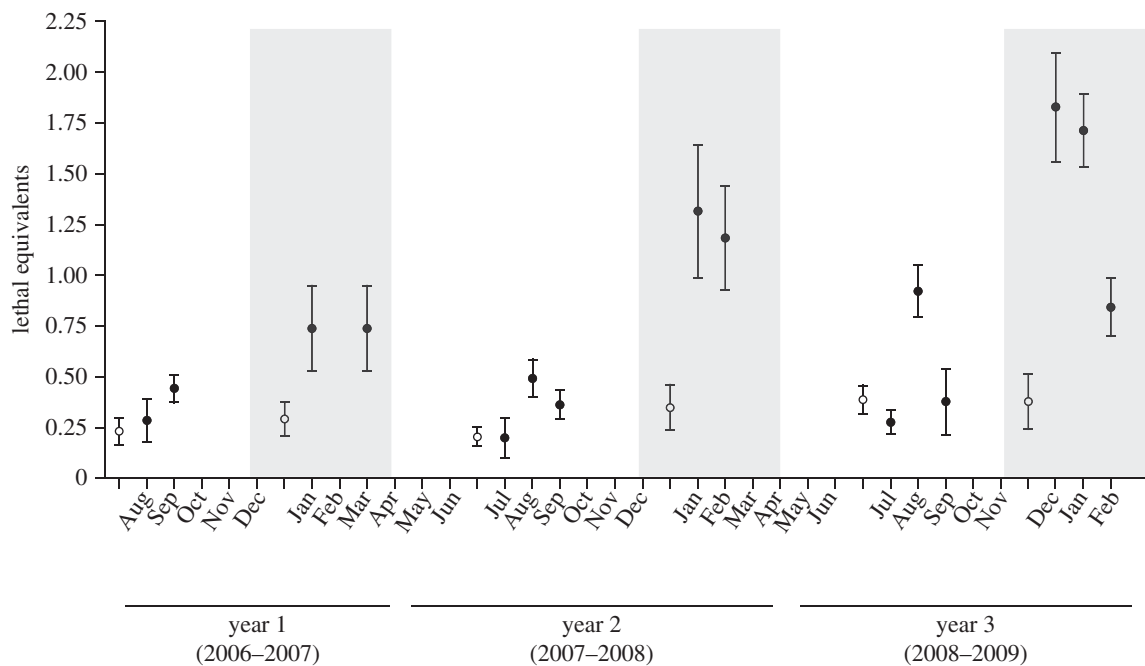


Figure 1. Inbreeding depression (lethal equivalents) for population productivity under field and laboratory conditions. The number of offspring produced in a single generation was measured in outbred ($F=0$) and inbred ($F \approx 1$) populations during both winter (shaded) and summer (clear) months in the field (filled circles) and in the laboratory (open circles).

Table 2. Analysis of inbreeding depression observed in the field and laboratory. ANOVA compared the number of lethal equivalents (β) expressed under three environmental conditions (ENV): in the field during the summer; in the field during the winter; and under controlled laboratory conditions over 3 years (YEAR) in six separate inbred lines (LINE). Interactions with $p > 0.25$ were removed from the model.

source	d.f.	ms	F -value	p -value
ENV	2	9.340	37.063	<0.0001
YEAR	2	0.836	3.317	0.041
LINE	5	0.428	1.698	0.143
error	106	0.252		

stress patterns observed. The average number of lethal equivalents was more than twofold higher during winter months relative to summer months (1.20 versus 0.45, $p < 0.001$; table 3). In contrast, the high temperatures prevailing during summer field conditions did not significantly amplify inbreeding depression relative to benign laboratory conditions (table 3).

When the field data for stress level and inbreeding depression were compared, we found a significant positive linear relationship (figure 2; slope = 1.56 ± 0.39 and 1.57 ± 0.23 for unconstrained and constrained analyses, respectively), explaining approximately 60 per cent of the variance ($r^2 = 0.59$; $p < 0.001$), which translates to an additional 0.16 lethal equivalents expressed for every 10 per cent increase in the stress level. ANCOVA was initially run including all possible interactions of STRESS LEVEL with SEASON and YEAR; none of them were significant ($p > 0.10$). After removal of these interactions, only the covariate STRESS LEVEL was significant ($p < 0.001$); the effects of SEASON, YEAR and SEASON \times YEAR were all non-significant ($p > 0.10$).

Table 3. The expression of lethal equivalents in different environments. (a) The average number of lethal equivalents (β_{avg}) in each environment and (b) the difference in lethal equivalents between environments (β_{diff}) compared using Tukey's HSD post hoc test.

(a) environment	$\beta_{\text{avg}} \pm \text{s.e.}$
laboratory	0.311 ± 0.04
field summer	0.446 ± 0.05
field winter	1.203 ± 0.14
(b) post hoc comparison	$\beta_{\text{diff}} \pm \text{s.e.}$
summer versus laboratory	0.135 ± 0.11
winter versus laboratory	$0.892 \pm 0.12^{***}$
winter versus summer	$0.754 \pm 0.11^{***}$

*** p -value < 0.001.

Adding nonlinear quadratic and cubic terms did not significantly improve model fit relative to the first order linear regression ($p > 0.05$). In this analysis, the values within a given season are not strictly independent because a single set of laboratory data was used as the 'benign' reference (figure 1). However, this lack of independence did not affect the results because (i) analysis of data based on the seasonal averages for each year (six data points) resulted in a slope of 1.55 ± 0.45 ($p < 0.05$); and (ii) using the average of all the laboratory values to calculate stress levels and $\Delta\beta$ for all seasons and years (15 data points) yielded a slope of 1.57 ± 0.25 ($p < 0.001$). The average outbred productivity in the laboratory was 516, with an average inbreeding depression (β) of 0.31.

The slope of 1.56 estimated under field conditions was not significantly different from the slope of 2.08 ± 0.47 (unconstrained) estimated using data from published studies of *Drosophila* laboratory populations (figure 2), because inclusion of StudyType (the present study versus published laboratory data) in the ANCOVA did

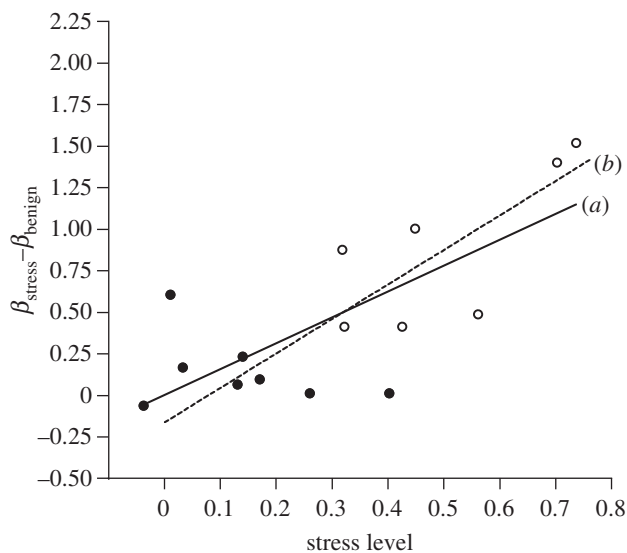


Figure 2. The relationship between stress level and the associated change in the level of inbreeding depression, defined as the difference in lethal equivalents expressed under stressful (β_{stress}) and benign (β_{benign}) conditions. (a) Data points from the current field experiment with the fitted regression line ($\Delta\beta = 0.005 + 1.56 \text{ stress level}$), where stress and benign correspond to field and laboratory, and (b) the regression line fitted to published data from laboratory populations of *D. melanogaster* and *D. buzzatii* under different abiotic and biotic stresses (see electronic supplementary material, table S1; $\Delta\beta = -0.08 + 2.08 \text{ stress level}$). Filled circles denote summer; open circles denote winter.

not significantly affect the slope (StudyType \times Stress-Level, $F_{1,33} = 0.858$ $p > 0.25$). Finally, as did Fox & Reed [5], we confirmed that this significant positive relationship was not driven by the inherent dependence of both stress level and inbreeding depression on the fitness of outbred individuals. We found that stress level and inbreeding depression were on average uncorrelated (slope = 0.05 ± 0.05) across 10 randomly generated datasets (F level = 0.25 or 1.00, five datasets each) sampled from a uniform distribution bounded by reported levels of stress and inbreeding depression in *Drosophila* studies (see §3).

(c) Temperature measurements in the field

While laboratory populations were kept at a constant 25°C, the field populations experienced an overall average (\pm s.d.) temperature of 22°C \pm 4.3 during the three summer seasons and 12°C \pm 2.7 during the three winter seasons (table 4). The overall average daily high (\pm s.d.) and low (\pm s.d.) were 28°C \pm 2.8 and 18°C \pm 2.2 during the summer, and 16°C \pm 3.7 and 10°C \pm 2.5 during the winter. Peak temperatures (highest recorded temperature for a season) during the summer experiments averaged 33°C (range across years: 31–35°C) while the minimum temperatures (lowest recorded temperature for a season) during the winter experiments averaged 6°C (range across years: 4–8°C).

4. DISCUSSION

Our 3-year field study (2006–2009) using captive populations of *D. melanogaster* is the first field study to

Table 4. Summary of temperature data (avg \pm s.d.) collected in the field during (a) summer and (b) winter seasons (temperature in °C was recorded hourly using iButtons placed next to experimental bottle populations).

	average daily temperature	average daily high	average daily low	days sampled
(a) summer				
year 1	23 \pm 1.5	29 \pm 2.4	19 \pm 1.7	75
year 2	21 \pm 3.0	28 \pm 4.6	17 \pm 2.2	79
year 3	21 \pm 3.0	27 \pm 2.5	17 \pm 2.4	74
seasonal average	22 \pm 4.3	28 \pm 2.8	18 \pm 2.2	76 \pm 1.5
(b) winter				
year 1	14 \pm 2.5	18 \pm 3.4	11 \pm 2.5	85
year 2	10 \pm 1.6	13 \pm 2.2	8 \pm 1.6	76
year 3	12 \pm 2.3	16 \pm 3.5	9 \pm 2.0	76
seasonal average	12 \pm 2.7	16 \pm 3.7	10 \pm 2.5	79 \pm 3.0

investigate the relationship between inbreeding depression and seasonal fluctuations in stress level. We found that stress levels were on average fourfold higher in the winter compared with the summer, and that this higher stress level increased the inbreeding depression affecting population productivity from 32 per cent in the summer to 65 per cent in the winter. Moreover, this covariation of stress level and inbreeding depression conformed to the same linear relationship seen in experimental *Drosophila* laboratory studies of stress and inbreeding (figure 2). The observation that inbreeding depression based on population productivity in the field follows the same relationship as inbreeding depression based on larval survival in the laboratory suggests that even in complex environments stress and inbreeding interact in the same predictable manner. This same general relationship has been found in laboratory studies across a variety of species [5,13].

Heat is commonly thought to be highly stressful owing to negative effects on the proper folding and functioning of proteins [3,30,34], and as a result the effects of heat stress on inbreeding depression have received considerable attention [5,9]. However, it has been suggested that winter is the most stressful season for *D. melanogaster*, as evidenced by marked reductions in population size relative to other seasons [35,36]. We found that the relatively mild winter temperatures of southern California were, for *D. melanogaster*, highly stressful, while summer conditions imposed little cost to population productivity relative to benign laboratory conditions (figures 1 and 2). The thermal range of *D. melanogaster* is 11–32°C, with viability decreasing sharply above and below the extremes [37–39], indicating both high and low temperatures can be stressful. In the current study, average daily summer temperatures experienced by *D. melanogaster* populations were close to optimal, whereas winter average temperatures were close to lethal extremes, and daily lows frequently went below physiological limits for growth and reproduction (table 4). Cold temperatures may be costly for population productivity by inducing reproductive diapause [40,41] or by slowing the growth of larvae. Sufficiently slow larval growth is probably lethal under natural conditions since the food resource rots away, so it is not

surprising that larval diapause has not been reported. The tropical origins of this species [42] may in part explain why conditions closer to the upper limits of heat tolerance (at or near 32°C) were less stressful. It may also account for the apparent absence of pupal diapause in this species. Other aspects of seasonality, such as photoperiod, may also contribute to differences in stress levels between summer and winter by affecting the induction of reproductive diapause in females, thus reducing population productivity [41], but it is unknown if this would differentially affect inbred flies.

In evaluating the levels of inbreeding depression observed in the laboratory versus field, we need to consider any potential bias arising from creating inbred lines in the laboratory using a fast rate of inbreeding (low effective population size). Such rapid inbreeding is predicted to purge lines of highly deleterious alleles, which could lead to conservative estimates of inbreeding depression. However, this effect is likely to be roughly equal across both field and laboratory environments since such alleles are likely to have similar lethal or near-lethal effects in both environments. Any additional effects of purging under the benign rearing environment used to create inbred lines are not expected to influence levels of inbreeding depression under field conditions in a predictable way, as it is known the effects of purging can be both environment- and trait-specific [17,43–45].

Among previous *Drosophila* laboratory studies, stress level was largely uncontrolled and varied as much as threefold across stress types [10,30,46,47], creating the impression that inbreeding and stress interact in an unpredictable manner because the data were analysed in terms of the categorical variables ‘stress’ versus ‘benign’ [11,48,49]. Furthermore, the expression of inbreeding depression is commonly thought to be specific to the environment and genetic background of a population [9,11,49]. However, measuring stress as a continuous variable produced a general linear relationship between stress level and stress-induced inbreeding depression [5], which conflicts with these assumptions, especially in light of the variable species and stressful environments included in these datasets. On the other hand, it is well established that global stress–response ‘programmes’ [50] involving both stress-induced and stress-repressed genes exist for many organisms, and work in both yeast and *Drosophila* has shown that various abiotic and biotic stress types evoke similar changes in biochemical pathways at the cellular level [51,52]. If the expression of genetic load adversely affects the ability of inbred individuals to mount fundamental core stress response mechanisms, then this could result in similar levels of inbreeding depression across many different stress types. However, the underlying genetic architecture responsible for inbreeding–stress interactions is unknown [53,54]. Although recent work in *Drosophila* has demonstrated a detectable molecular fingerprint of inbreeding on gene expression [46,51,55,56], it will be important to determine whether these patterns are unique to inbreeding or are in fact also seen in outbred populations exposed to higher levels of the same stress. Finally, there is increasing evidence that inbreeding depression is in part due to loci demonstrating a threshold effect, whereby neutral or beneficial alleles become deleterious under environments or at certain stress levels [4]. Therefore, in contrast to

cases where many loci contribute to the expression of inbreeding depression, a strong linear relationship between stress and inbreeding depression may not hold for individual studies and stressors where few loci are involved and threshold effects of deleterious alleles could contribute to departures from linearity. Because the underlying genetic architecture of inbreeding depression is expected to be both trait- and population-specific, it is unknown how prevalent threshold effects might be.

Recognizing a general linear relationship between stress levels and the expression of inbreeding depression has implications for evolutionary and conservation biology. Understanding inbreeding–stress interactions under natural conditions is an important step in addressing the broader question of how temporal variation in environmental conditions interacts with inbreeding in small populations to affect survival and extinction risk [2,16,43,47]. Conservation biologists are often faced with managing small populations predicted to experience both increased inbreeding and environmental stress in the form of global climate change, habitat destruction and pollution. Identifying the most stressful conditions and/or times of year for these populations could aid in predicting when extinction risk is heightened owing to inbreeding–stress interactions. Both population viability simulations [8] and empirical work in *Drosophila* [43,47] demonstrate that interactions between stress and inbreeding negatively impact population dynamics by significantly increasing extinction risk in small populations. However, only recently have inbreeding–stress interactions been incorporated into simulations that estimate persistence times of populations [8]. From a conservation standpoint, an important next step is to quantify the effects of inbreeding–stress interactions on the persistence of wild populations by measuring both stress levels and inbreeding depression under natural conditions. Such information can then be incorporated into the development of more accurate population viability and extinction analyses of many species predicted to suffer the consequences of anthropogenically induced climate change.

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