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Environmental Stress and The Expression of Inbreeding Depression in *Drosophila melanogaster*

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by

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ABSTRACT OF THE DISSERTATION

Environmental Stress and The Expression of Inbreeding Depression in *Drosophila melanogaster*

by

Laramy Sherian Enders

Doctor of Philosophy, Graduate Program in Evolution, Ecology and Organismal Biology
University of California, Riverside, March 2011
Dr. Leonard Nunney, Chairperson

Exposure to environmental stress is a common feature of the life of most organisms, making the ability to cope with such stress imperative to survival and persistence. In particular, small inbred populations that suffer the deleterious effect of inbreeding depression may be particularly vulnerable to the effects of environmental stress. However, we lack an understanding of the factors that contribute to the considerable variation in the effects of stress on the expression of inbreeding depression. In this dissertation I examined the role of four factors in determining the outcome of inbreeding-stress interactions.

First I investigated the relationship between stress level, measured as the reduction in fitness a stress causes relative to benign conditions, and the

magnitude of inbreeding depression. I found a strong positive linear relationship both in laboratory populations and populations exposed to field conditions, whereby inbreeding depression increases as the level of stress increases. Contrary to initial predictions, in a three year field study I found winter conditions were more stressful and induced greater inbreeding depression than summer conditions.

Second, I examined how different types of abiotic (heat and ethanol) and biotic (larval competition and pathogenic bacteria) stresses affect the expression of inbreeding depression both during exposure (larval survival) and after exposure (male mating). I found that during exposure stress amplified inbreeding depression relative to benign conditions, but the effect was only significant for heat and competitive stress. In addition, I found that exposure to stress during development reduced inbreeding depression for competitive male mating success, an effect that was significant for the two biotic stresses.

Finally, I investigated how stress and sexual selection affect the expression of inbreeding depression in males versus females. I found a striking difference in the magnitude of inbreeding depression expressed in the sexes, with males suffering a two-fold higher cost to being inbred that females. This is presumably due sexual selection, via female choice and/or male competitive interactions, increasing selection against inbred males expressing deleterious alleles.

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Introduction

Currently, stress is most widely considered to be any environmental factor that significantly reduces the fitness of an individual relative to more benign conditions (Hoffmann & Parsons, 1991; Bijlsma & Loeschcke, 2005). Natural populations are constantly challenged by abiotic and biotic environmental stresses in the form of pollution, climate change, disease, and increased competition for resources. Stress is therefore recognized as having major implications for both short-term survival and long-term adaptation (Loeschcke *et al.*, 2004). In particular, environmental stress is recognized as influencing the survival of small populations that experience inbreeding and inbreeding depression (Bijlsma *et al.*, 2000; Reed *et al.*, 2002; Liao & Reed, 2009). As a result, the role of environmental stress in determining population persistence has become a major concern in conservation biology.

Central to conservation biology is the idea that small populations suffer a higher risk of extinction due to the negative consequences that result from random genetic drift and inbreeding primarily determining the dynamics of genetic variation (Bouzat, 2010; Ouborg *et al.*, 2010). Individuals may be inbred because their parents are close relatives or because they are members of a small population where all individuals share a high degree of coancestry. In either case, increased homozygosity due to inbreeding increases the expression of recessive deleterious or lethal alleles, which can lead to a reduction of fitness known as inbreeding depression (Charlesworth & Willis, 2009; Kristensen *et al.*,

2010). Environmental stress is predicted to exacerbate this problem by increasing the number of deleterious alleles expressed and/or amplifying the negative effects these alleles have on fitness (Bijlsma *et al.*, 1999; Dahlgaard & Hoffmann, 2000; Haag *et al.*, 2002; Reed *et al.*, 2003). It has been shown that the deleterious effects of stress are often amplified in inbred individuals (Crnokrak & Roff, 1999; Armbruster & Reed, 2005), rendering small inbred populations particularly vulnerable to such effects (Bijlsma *et al.*, 2000; Reed *et al.*, 2002). However, the magnitude of inbreeding depression expressed under stressful conditions varies considerably (Armbruster & Reed, 2005), suggesting additional factors may be important in explaining how stress and inbreeding interact in small populations (Hedrick & Kalinowski, 2000; Armbruster & Reed, 2005; Waller *et al.*, 2008; Fox & Reed, 2010).

The purpose of this dissertation is to investigate factors that influence the relationship between inbreeding and environmental stress using the model system *Drosophila melanogaster*. Specifically, I explore the role of: 1) Stress Level 2) Stress Type 3) Timing of Exposure to Stress and 4) Sexual Selection. *D.melanogaster* is ideal for manipulation of inbreeding levels and provides efficient lab rearing where environmental conditions are easily manipulated. I was also able to utilize wild-caught populations of *D.melanogaster* from California, which have a genetic architecture unmodified by laboratory rearing.

In chapter one, I examine the relationship between inbreeding depression and stress level. The magnitude of effect a specific stress has on fitness (stress

level) can be measured as the reduction in fitness of healthy outbred individuals relative to benign conditions. A recent meta-analysis showed that there is a strong linear relationship between stress level quantified in this way and inbreeding depression measured as lethal equivalents (Fox & Reed, 2010). The aim of my first chapter was to examine the generality of this result by first determining if a linear relationship holds for the specific case of stress applied in the lab on larval survival in *Drosophila*, using data not included in the Fox and Reed (2010) study. Second, I tested this relationship using data from a 3-year field study, where seasonal variation in temperature and humidity imposed stress on the productivity of small bottle populations of *D. melanogaster.* My initial prediction was that stress would be high in the hot Southern California summer and minimal during the mild winters. Results showed a strong positive linear relationship between inbreeding depression and stress level under both field and laboratory conditions, which translated to an additional 0.17 lethal equivalents expressed for every 10% increase in stressfulness. Contrary to the expectation that summer heat stress is most harmful for the productivity of field populations, I found that cold winter temperatures were the most stressful and induced the greatest levels of inbreeding depression. The results of this chapter suggest the effect of stress on inbreeding depression is not specific to the genetic architecture of a population and/or stress type, but rather general molecular or biochemical mechanisms may underlie stress-inbreeding interactions. Overall, stress level explained approximately half of the variance in inbreeding depression

expressed under stressful conditions (r^2 = 0.52), indicating additional factors, such as stress type and timing of exposure, may contribute to the remaining unexplained variance.

In chapter two of this dissertation I focus on examining how the type of stress and the timing of exposure to stress affect the expression of inbreeding depression. It is currently unknown if fundamental differences in the way in which stressors affect the expression of genes and hence the expression of genetic load translate into qualitatively different outcomes for the survival of inbred individuals. In addition, variation in levels of inbreeding depression under different types of stress may depend on timing of exposure to stress (i.e. developmental period at which individuals experience stress). It is unknown whether stress has long lasting phenotypic effects on an individual that will lead to enhanced inbreeding depression across multiple life history stages. Stress may also increase selection against deleterious mutations that affect fitness at multiple life-history stages, and therefore could purge genetic load and reduce the magnitude of inbreeding depression in later life history stages (Enders & Nunney, 2010).

In the second chapter I standardized the level of stress, which allowed a direct comparison of the effect of several abiotic and biotic stressors on the magnitude of inbreeding depression expressed both during and after exposure (larval survival and male mating, respectively). Results showed that not all stress types amplify inbreeding depression during exposure relative to benign

conditions. Heat and larval competitive stress caused the highest inbreeding depression for larval survival, while bacterial and ethanol had little effect. In addition, I found evidence that males exposed to stress during development had significantly lowered inbreeding depression for mating success relative to males that experienced benign conditions. However, only the two biotic stresses show a significant negative relationship between the level of inbreeding depression during exposure (larval survival) and post-exposure (male mating). This relationship is expected if purging of less fit larvae occurs during the stressful developmental stage.

Finally, in chapter three, I test whether sexual selection in the form of female choice and/or male-male competition leads to sex-specific differences in the magnitude of inbreeding depression expressed in wild caught *D. melanogaster*. In general, the cost of inbreeding on fitness varies considerably across life history traits (Miller & Hedrick, 1993; Crnokrak & Roff, 1999; Charlesworth & Willis, 2009; Robinson *et al.*, 2009). However, a review of the *Drosophila* literature suggested that inbreeding depression may be greatest for males, possibly due to increased selection against deleterious alleles resulting from female choosiness and mate competition (Whitlock & Bourguet, 2000; Whitlock & Agrawal, 2009). The aim of this chapter was to test the hypothesis that sexual selection exacerbates inbreeding depression in inbred males and therefore females bare a relatively lower cost to being inbred than males do. In addition, I explored the effects of larval competitive stress on egg hatchability,

larval survival, male mating and female fecundity. Overall, I found that one generation of sibling mating resulted in a substantial cumulative fitness loss (egg to adult reproduction) of 50% under benign conditions and 73% under increased larval competition (stressed). In addition, inbred male offspring suffering a 2-fold higher reproductive cost than females, independent of stress. This suggests that sexual selection exacerbates inbreeding depression when incorporated into the measure of adult fitness (competitive male mating success) versus when it is not (female fecundity measured as number of offspring produced).

This dissertation demonstrates a number of important features of the relationship between inbreeding and fitness under stressful environmental conditions. First, the magnitude of the cost to fitness (stress level) induced by a stressor was found to be a reliable predictor of increased inbreeding depression under stressful relative to benign conditions. Second, when stress levels were standardized it was found that not all stresses induce equivalent levels of inbreeding depression, indicating that stress type may be an important determinant of the outcome of stress-inbreeding interactions. Third, we found evidence that stress experienced early in development can act to purge genetic load and reduce inbreeding depression in later life history stages, but only for certain stress types. Finally, we find a striking difference in the magnitude of inbreeding depression expressed in males relative to females, presumably due to increased selection via female choice against recessive deleterious alleles expressed in males.

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Chapter 1

Stress and Inbreeding: A General Rule Predicts the Effects of Seasonal Stress on Inbreeding Depression in Captive Field Populations of *Drosophila melanogaster*

Abstract

Understanding the way in which environmental stress exacerbates the deleterious fitness consequences associated with inbreeding has implications for a wide range of research topics including the genetics of the stress responses of individuals, the evolution of inbreeding avoidance, and the persistence of small populations. A recent meta-analysis conducted across a broad range of taxa demonstrated a strong linear relationship between the change in the magnitude of inbreeding depression under stress and the stress level, measured as fitness loss in outbred individuals. We confirmed with independent published data that a similar linear relationship holds in *Drosophila* laboratory populations exposed to a single stressor, a result unaffected by the type of stress. To test the generality of this result under more complex conditions, we analyzed productivity data from a field study of single-generation captive populations of inbred and outbred *Drosophila melanogaster*, replicated over 3 years of seasonal change. Contrary to the prediction that summer heat stress is most harmful for population productivity, we found that cold winter temperatures were most stressful and induced the greatest levels of inbreeding depression. We found that the same linear relationship applied to both laboratory and field data, with a joint slope of 1.71, i.e. an additional 0.17 lethal equivalents were expressed in the inbred lines for every 10% increase in stress level. Combining these data with those of the prior study increased the slope slightly to 2.29. By reformulating this general result, we propose that it arises because the effect of a given stress on inbred

lines is qualitatively similar to the effect on outbred lines, but that the effect is amplified in a predictable manner proportional to the level of inbreeding.

Introduction, Results and Discussion

Wild populations are routinely subjected to a range of environmental challenges, including climate change, destruction and loss of habitat, pollution, and the introduction of invasive species, that threaten survival and persistence (Hoffmann & Parsons, 1991; Imasheva & Loeschcke, 2004; Frankham, 2005; Liao & Reed, 2009). Environmental threats such as these are commonly thought to magnify the deleterious fitness consequences that result from inbreeding (Fox & Reed, 2010; Fox *et al.*, 2010). However, considerable variation in the effects of stress on inbreeding depression has been documented, suggesting additional factors may be important in explaining how stress and inbreeding interact in small populations (Armbruster & Reed, 2005; Waller *et al.*, 2008; Fox & Reed, 2010). Armbruster and Reed (2005) reported that on average inbreeding depression increases when individuals are exposed to stressful relative to benign conditions. However, less than half (48%) of the 53 cases included in the review showed a statistically significant increase and 24% of cases actually report a decrease in inbreeding depression under stress, a pattern that has fueled continued controversy over the interaction between inbreeding and stress (Hedrick & Kalinowski, 2000; Armbruster & Reed, 2005; Waller *et al.*, 2008).

In general, stress is considered to be any environmental factor that significantly reduces the fitness of the individual or population relative to more benign conditions (Imasheva & Loeschcke, 2004; Bijlsma & Loeschcke, 2005). The magnitude of effect a specific stress has on fitness, or stress level, can therefore be measured as the reduction in fitness caused by a particular stress relative to benign conditions. Fox and Reed (2010) showed in their meta-analysis that there is a strong linear relationship between stress level quantified in this way and inbreeding depression measured as lethal equivalents. Lethal equivalents estimate inbreeding depression and are measured as the slope of the relationship between the natural log of fitness and the level of inbreeding (F) (Morton *et al.*, 1956). This result suggests that without standardization of stress levels within or across studies it is impossible to tease apart of effect of factors such as stress type or timing of exposure on stress-inbreeding interactions. For example, among *Drosophila* studies, differences in stress level vary as much as three fold across stress types (Bijlsma *et al.*, 2000; Reed *et al.*, 2002; Kristensen *et al.*, 2003; Kristensen *et al.*, 2008), creating the impression that inbreeding and stress interact in an unpredictable manner (Armbruster *et al.*, 2000; Hedrick & Kalinowski, 2000; Keller & Waller, 2002).

The results of Fox and Reed (2010) showed that the increase in inbreeding depression under stress is directly related to the magnitude of the stress and that the type of stress is relatively unimportant. Here we focus on examining the generality of this result by first determining if a linear relationship

holds for the specific case of stress applied in the laboratory on larval survival in *Drosophila*, using data not included in the Fox and Reed (2010) study. We then tested this relationship using data reflecting a much more complex array of stresses than had been imposed in any of the laboratory studies. Specifically, we used data from a 3 year field study, where seasonal differences imposed unpredictable stresses on the productivity of small one-generation bottle populations of *Drosophila*. Our expectation when the study was initiated was that stress and inbreeding depression would be high in the hot Southern California summer and minimal during the mild winters. These predictions proved to be entirely wrong.

Stress Level and Inbreeding Depression in Laboratory Populations

Our first goal was to define the empirical relationship between inbreeding depression and stress in *Drosophila* and to determine if this was identical to that found by Fox and Reed (2010). 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 (Supplementary Table 1.1). We found a strong positive relationship (slope =2.08 $\pm$ 0.47, $p < 0.001$), explaining half of the variance ($r^2 = 0.50$), that demonstrates increasing the level of stress by 10% results in fitness reductions of 0.21 lethal equivalents for inbred individuals relative to outbred individuals (Figure 1.1a). Eliminating the 8 cases included in the study of Fox and Reed (2010) has little

effect on the resulting slope (2.05 ± 0.63). Like Fox and Reed (2010) we confirmed that this significant relationship was not driven by the inherent dependence of both stress level and inbreeding depression on the fitness of outbred individuals. These variables are either negatively correlated or uncorrelated in randomly generated data sets sampled from the real *Drosophila* dataset (Supplementary Table 1.2). Finally, we did not find evidence that the stress type (biotic, chemical, cold, heat; see Supplementary Table 1.1) influences the relationship between inbreeding depression and stress level, since the Stress Type x Stress Level interaction was far from significant ($F_{3,14}$ = 0.655 p >0.25).

Stress Levels and Inbreeding Depression in Field Populations

We conducted a three year field study (2006-2009) to test whether the linear relationship between stress level and inbreeding depression found in laboratory populations of *Drosophila* holds for populations experiencing seasonal fluctuations in the wild. We measured the productivity (number of offspring produced in a single generation) of inbred and outbred populations of *Drosophila melanogaster* under natural conditions in an orange grove in Southern California during both the winter and summer seasons.

Natural populations face complex environments where multiple stressors interact to affect both the short-term survival and long-term persistence. Exposure to multiple abiotic and biotic stresses may significantly alter inbreeding-environment interactions due to synergistic effects of combined stresses on

survival (Mittler, 2006). Only a handful of studies have measured temporal variation in levels of inbreeding depression in wild populations (Keller *et al.*, 2002; Hayes *et al.*, 2005; Marr *et al.*, 2006; Szulkin & Sheldon, 2007). 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 (Keller *et al.*, 2002), while in song sparrows periods of rain were shown to significantly increase inbreeding depression for hatching success (Marr *et al.*, 2006). However, there are no data quantifying the temporal fluctuations in levels of stress occurring in the field and linking these levels to inbreeding depression. Our field experiment was designed to provide such data.

We compared levels of inbreeding depression under field conditions in winter (Dec-March) and in summer (June-Oct) to levels observed under benign lab conditions. The laboratory populations were kept at constant 25°C, while the field populations experienced average daily high/low of 27/18°C during the summer and 16/10°C during the winter. As noted earlier, our expectation was that that inbreeding depression would be greatest during the hot Southern California summer, since heat is commonly thought to be highly stressful due to negative effects on the proper folding and functioning of proteins (Lindquist, 1986; Hoffmann & Parsons, 1991; Kristensen *et al.*, 2008). Peak temperatures during the summer experiments averaged 33°C while the minimum temperatures during the winter experiments average 6°C. The thermal range of *D.*

melanogaster is 11-32°C, with viability decreasing sharply above and below the extremes (David *et al.*, 2005; Trotta *et al.*, 2006; Hoffmann, 2010).

Contrary to the view that heat stress is extremely detrimental for population survival (Sorensen *et al.*, 2003; Kotak *et al.*, 2007; Kristensen *et al.*, 2008), we found that over 3 seasonal cycles the cold winter months were more stressful for populations of *D. melanogaster* than summer ($F_{1,13}$ = 20.14 p = 0.001, Figure 1.2). Winter months were on average 76% more stressful than summer months ($F_{1,13}$= 20.14 p = 0.001), with outbred populations producing 50% fewer offspring under winter field conditions relative to benign lab conditions, but only 12% fewer during the summer (Table 1.2). Note that our measure of stress controlled for the inevitable lowering of productivity per unit time at lower temperatures by measuring productivity over the period defined by larval development time.

Matching the stress patterns observed, the cold winter temperatures experienced in the field caused significantly greater inbreeding depression than summer heat (Table 1.1 and Figure 1.2). The number of lethal equivalents was more than 2 fold higher during winter months relative to summer months (1.20 vs 0.46, Table 1.1b). In contrast, the high temperatures prevailing during summer field conditions did not significantly amplify inbreeding depression relative to benign lab conditions (Table 1.1b).

When the field data for stress level and inbreeding depression were compared, we found a significant positive linear relationship (slope = 1.56 ± 0.39,

Figure 1.1b) explaining approximately 60% of the variance ($r^2 = 0.59$), that translates to an additional 0.16 lethal equivalents expressed for every 10% increase in the stress level. This relationship was not significantly different from the slope estimated using data from *Drosophila* laboratory populations (Figure 1.1b: Study Type x Stress Level, $F_{1,33} = 0.858$ $p > 0.25$). Using a weighted linear regression, we compared the combined *Drosophila* dataset of lab and field populations (slope = 1.71 ± 0.28) to that of Fox and Reed (2010). Our analysis showed that the linear relationship estimated for *Drosophila* in this study is not significantly different from that of the 27 species included in the meta-analysis by Fox and Reed (2010) (Study Type x Stress Level $F_{1,91} = 3.515$ $p = 0.06$), even when the 8 shared data points were removed from the *Drosophila* data set ($F_{1,83} = 2.78$ $p = 0.10$). However, it should be noted that both weighted and un-weighted linear regression of the dataset used in the Fox and Reed (2010) meta-analysis (slope = 2.90 ± 0.49 and 2.39 ± 0.50, respectively), resulted in a substantially smaller slope than they reported (slope= 3.69 ± 0.56). Combining all three of the datasets gives an overall relationship between stress level and change in inbreeding depression as: $(\beta_{stress}-\beta_{benign}) = -0.04 + 2.29$(*stress level)* that explains 34% of the variance ($r^2 = 0.34$) (un-weighted analysis). Note that, as found by Fox and Reed (2010), the y-intercept is not significantly different from zero (-0.04 ± 0.12); hence constraining the regression to pass through the origin has a negligible effect on the slope estimate. This slope translates to an increase of 0.23 lethal equivalents for every 10% increase in stress.

Three different datasets show a linear relationship between stress level and stress-induced inbreeding depression; moreover all three have a very similar slope. The generality of this linear relationship is surprising in light of the variable species and stressful environments included in these datasets. The expression of inbreeding depression is commonly thought to be specific to the environment and genetic background of a population (Hedrick & Kalinowski, 2000; Keller & Waller, 2002; Armbruster & Reed, 2005), so at first it seems surprising to find that inbreeding and stress interact in a predictable and directional manner, both in artificially created environments and when populations experience complex natural conditions. The consistency of the linear relationship between stress and inbreeding indicates there may be general mechanisms that govern inbreeding-stress interactions.

Fox and Reed (2010) hypothesize that the linear increase in inbreeding depression as the stress level increases may reflect an increasing general instability of metabolic and stress response pathways resulting from the expression of deleterious mutations in inbred individuals. However, it is unclear why this process would result in a linear relationship independent of the type of stress, since the level of stress is defined by the fitness change in outbred individuals.

An alternative hypothesis to account for this linear relationship between stress level and inbreeding depression is that inbreeding results in a quantitative rather than a qualitative shift in the effect of stress, i.e. that the response of an

inbred individual to stress is essentially the same as the response of an outbred individual exposed to a higher level of the same stress. The proposal can be justified by rearranging the general relationship:

$$\beta_{stress} - \beta_{benign} = bS \qquad (1)$$

where the two β-values define the lethal equivalents expressed under a stress level S and under benign conditions respectively, and b is the empirically defined slope (b≈2). To a good approximation, the general linear relationship (1) can be re-written as:

$$\left[\frac{w_{inbred|stress}}{w_{inbred|benign}}\right] \approx \left[\frac{w_{outbred|stress}}{w_{outbred|benign}}\right]^{(1+bF)} \qquad (2)$$

This formulation shows that the fitness of stressed inbred individuals relative to unstressed inbreds is directly related to the relative fitness of stressed and unstressed outbred individuals.

Any environmental challenge activates a set of genes specific to that stress that to some degree ameliorates its detrimental effects. Thus the measure "stress level" (the loss of fitness observed when the outbred population shifts from a benign to the stressful environment) is a measure of the failure of that fraction of the genome to achieve environmental buffering. Here we propose that the linear rule arises because, for a given stress, the same fraction of the genome is involved in this environmental buffering for inbred and outbred individuals, but the failure rate increases in a multiplicative (independent) way with increasing inbreeding.

If we assume that the fitness of outbred individuals exposed to a stress S relative to unstressed individuals can be approximated by the independent action of *n* genes, each with a selection coefficient of δ_S (where δ_S is small), then from (2) we have:

$$\left[\frac{w_{inbred|stress}}{w_{inbred|benign}}\right] \approx (1-\delta)^{n(1+bF)} \approx (1-[1+bF]\delta)^n$$

so the linearity could be generated by a progressive increase in the average selection coefficient with F across the n loci; however it remains unclear why the coefficient *b* would remain relatively constant across a broad range of taxa and stress conditions.

The underlying genetic architecture responsible for inbreeding-stress interactions is unknown (Kristensen *et al.*, 2010; Ouborg *et al.*, 2010). However, recent work in *Drosophila* has demonstrated a detectable molecular fingerprint of inbreeding on gene expression (Kristensen *et al.*, 2002; 2003; Kristensen *et al.*, 2005; Sorensen *et al.*, 2005a; Sorensen *et al.*, 2005b; Kristensen *et al.*, 2006). It will be important to determine if these patterns are unique to inbreeding or are in fact also seen in outbred populations exposed to higher levels of the same stress.

Recognizing a general relationship between stress levels and the expression of inbreeding depression has implications for evolutionary and conservation biology. We have demonstrated through a 3-year study of captive populations of *D. melanogaster* in the field, that seasonal variation in inbreeding

depression is directly linked to fluctuations in stress level. 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 (Bijlsma *et al.*, 1999; Reed *et al.*, 2002; Frankham, 2005). 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 due to inbreeding-stress interactions. Both population viability simulations (Liao & Reed, 2009) and empirical work in *Drosophila* (Bijlsma *et al.*, 1999; Reed *et al.*, 2002) 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 (Liao & Reed, 2009). 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 anthropogenic induced climate change.

Tables and Figures

Table 1.1: Analysis of inbreeding depression observed in the field experiment. a) ANOVA comparing the number of lethal equivalents (β) expressed in the field during the summer and during the winter, and under controlled laboratory conditions (ENV). Inbreeding depression (lethal equivalents) was calculated for six separate inbred lines (LINE) over three years (YEAR). Interactions with $p > 0.25$ were removed from the model. b) The average number of lethal equivalents (β_{avg}) in each environment, difference in lethal equivalents between environments (β_{diff}), and the significance of each comparison using Tukey's HSD post hoc test.

A) ANOVA for Lethal Equivalents

Source	DF	MS	F	p value
ENV	2	9.340	37.063	<.0001
YEAR	2	0.836	3.317	0.041
LINE	5	0.428	1.698	0.143
Error	86	0.252		

B) Average Lethal Equivalents and Post Hoc Comparisons

ENV	β_{avg} (se)	
Lab	0.311(0.04)	
Field Summer	0.446(0.05)	
Field Winter	1.203(0.14)	
Comparison	β_{diff} **(se)**	**p value**
Summer vs Lab	0.135(0.11)	0.469
Winter vs Lab	0.892(0.12)	<0.001
Summer vs Winter	0.754(0.11)	<0.001

Table 1.2: Analysis of seasonal variation in stress levels found in the field. a) Stress levels experienced during the winter and summer seasons of a 3-year field study that measured the productivity of inbred and outbred bottle populations of *D. melanogaster.* Stress level was calculated using the relative population productivity (number of offspring) of outbred (OB) populations under field and laboratory conditions: $1-(OB_{Field}/OB_{Lab})$. b) ANOVA comparing stress levels during the winter and summer (SEASON) across the 3 years (YEAR) of the field study.

A) Stress Levels

	Summer	Winter
Year 1		
Aug	0.32	
Sep	0.11	
Jan		0.39
Mar		0.48
Year 2		
Jul	0.04	
Aug	0.20	
Sep	0.23	
Jan		0.41
Feb		0.27
Year 3		
Jul	0.28	
Aug	-0.19	
Sep	-0.04	
Dec		0.72
Jan		0.68
Feb		0.53
Average Stress Level	0.12 (0.06)	0.50 (0.06)

B) ANOVA

Source	DF	MS	F	p value
SEASON	1	0.463	24.190	< 0.001
YEAR	2	0.054	2.838	0.101
Error	11	0.019		

Figure 1.1: 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) Laboratory populations of *D. melanogaster* and *D.buzzatii* under different abiotic and biotic stresses, using published data. b) Field populations of *D. melanogaster* during summer and winter seasons (current study). The level of stress is measured as the percent loss of fitness of outbred individuals under stressful relative to benign conditions.

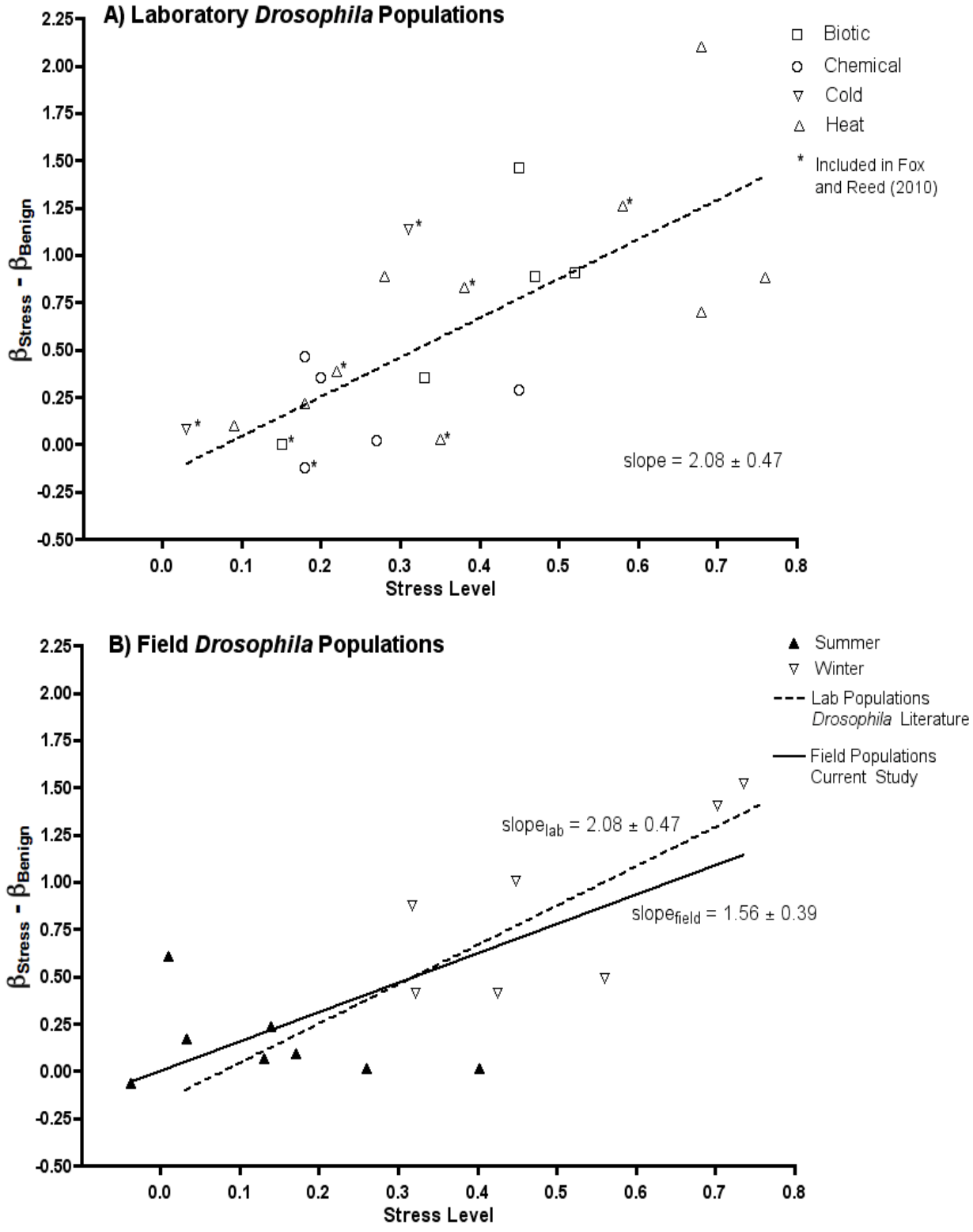
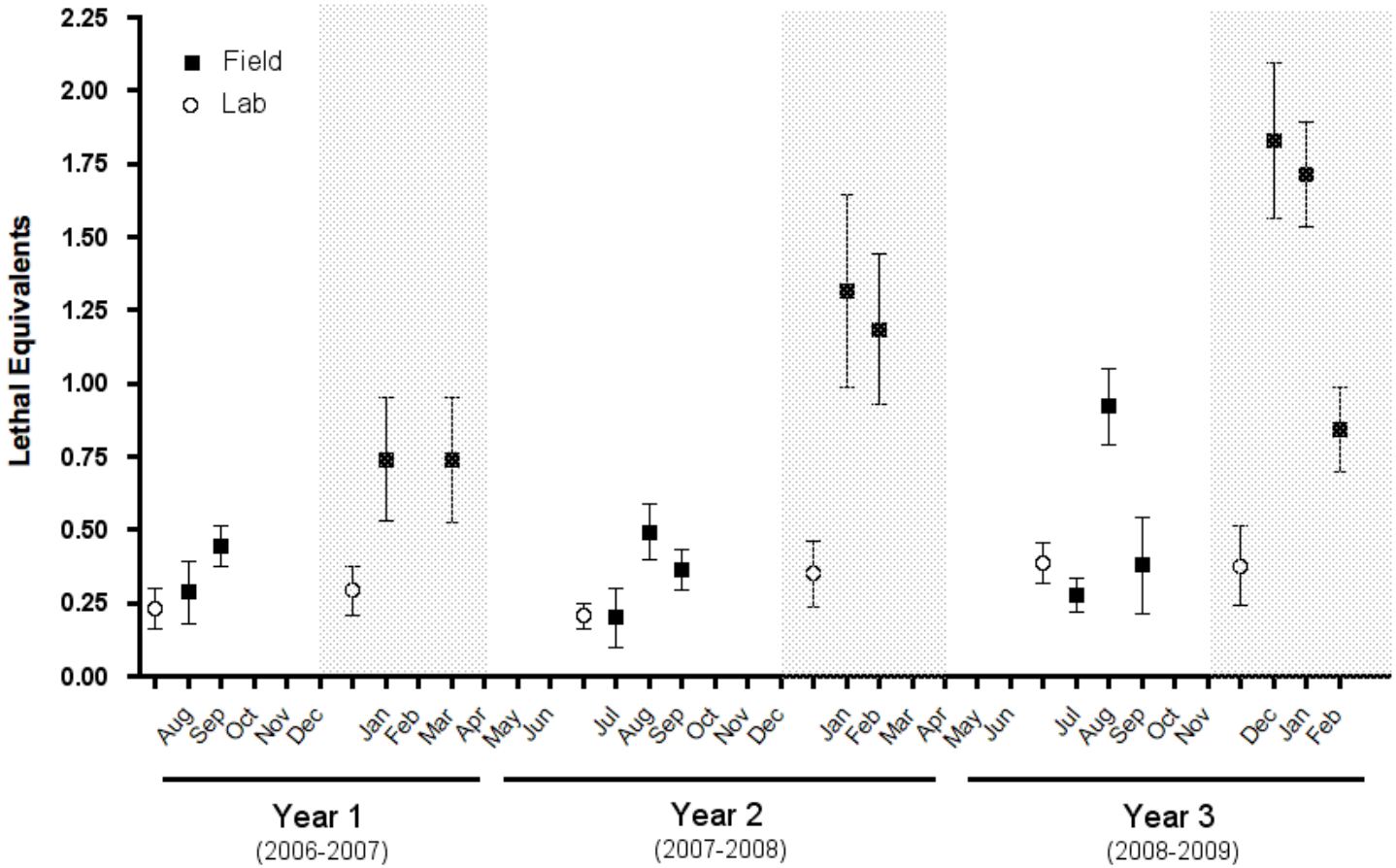

A) Laboratory *Drosophila* Populations
Biotic
Chemical
Cold
Heat
* Included in Fox and Reed (2010)
slope = 2.08 ± 0.47
$\beta_{Stress} - \beta_{Benign}$
Stress Level
B) Field *Drosophila* Populations
Summer
Winter
Lab Populations *Drosophila* Literature
Field Populations Current Study
$slope_{lab}$ = 2.08 ± 0.47
$slope_{field}$ = 1.56 ± 0.39
$\beta_{Stress} - \beta_{Benign}$
Stress Level

Figure 1.2: 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 ~1) populations during both winter (shaded) and summer (clear) months in the field (squares) and in the laboratory (circles).


Lethal Equivalents
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Field
Lab
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Year 1
(2006-2007)
Year 2
(2007-2008)
Year 3
(2008-2009)

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Methods

Stress Level and Inbreeding Depression: Laboratory Populations

We investigated the effect of stress level on the expression of inbreeding depression in stressful and benign environments using published work in *Drosophila.* We included only studies that measured egg to adult survival, used a single stressor and the stress level was positive (i.e. stressful conditions reduced fitness). Stresses included biotic forms such as intraspecific larval competition, temperature (hot and cold), and various types of chemicals. A linear regression was performed on the difference between lethal equivalents expressed under benign and stressed conditions (β_{stress} - β_{benign}) and the level of stress. Stress level was calculated as described by Fox and Reed (2010):

1- ($Survival_{outbred\_stress}$/$Survival_{outbred\_benign}$).

Lethal equivalents used were those published by Armbruster and Reed (2005) with the exception of four studies published after 2005 (Ferriera & Amos, 2006; Kristensen *et al.*, 2008; Enders & Nunney, 2010; Mikkelsen *et al.*, 2010) and one unpublished study(Enders & Nunney, in prep). Supplementary Table 1.1 provides information on the studies used in the analysis.

Stress level and inbreeding depression are both measured using the survival of outbred individuals and are therefore not independent measures. To measure the magnitude of the inherent correlation between these measures, we randomly generated 10 data sets 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®). The simulated fitness values were used to calculate stress level and the number of lethal equivalents. A linear regression was then run on the difference in lethal equivalents under stressful and benign conditions (β_{stress} - β_{benign}) and stress level. Based on reported levels of stress and inbreeding depression in *Drosophila* studies, the upper bounds of these measures were set at 75% (stress level) and 50% (inbreeding depression). Two levels of inbreeding depression (F level = 0.25 or 1.00) were used in separate simulations (5 per F level).

Stress Level and Inbreeding Depression: Productivity of Field Bottle Populations

Inbred populations were created through 20 generations of full-sibling mating from the offspring of single females collected in the University of California, Riverside campus orange grove. These lines were considered to be completely homozygous (level of inbreeding, F ~1). Outbred populations were created by combining an equal number of individuals from each of 10 inbred lines. From these inbred and outbred stock populations we created experimental bottle populations of equal density (30 males: 30 females) that were placed simultaneously in the laboratory and the field during the winter and summer seasons from 2006-2009. Populations were placed together under a single tree in the orange groves located on the campus of the University of Riverside, California (field) or in an incubator set at a constant temperature of 25C (lab). Sets of these bottles, consisting of 3 replicates per 6 inbred lines and 3 outbred lines, were simultaneously placed in the field and lab incubator during the three

hottest (July, August, September) and three coldest months (December, January, and Februrary). 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 (ibutton ®Dallas Maxim data loggers) placed with the experimental bottles.

Bottles placed in the field contained 50ml of standard cornmeal molasses media and were covered with four layers of cheesecloth to allow air flow. A custom made device made from a small aluminum rod was fitted to the opening of the bottles and served as a stopper to prevent food ,detached from the bottom of the bottle, from trapping or killing emerged flies when they were collected. Each bottle was then placed in a separate plastic container, 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. Bottles placed under laboratory conditions contained the same amount and type of media but had foam plugs and no aluminum stopper inside.

The population productivity of each bottle population was measured as the total number of progeny produced in a single generation. Population productivity is a measure that reflects the product of fecundity, fertility, and survivorship, a function that is closely related to fitness (Roper *et al.*, 1993; Fowler & Whitlock, 1999) and is used to estimate short-term population persistence. To ensure that productivity included only the first generation of offspring, a control bottle was set

up with at the time the first pupae were observed. This control bottle represents the ideal conditions under which a second generation could develop and thus when dark pupae were observed in the control bottles this marked the end of offspring collection. Adults were left in the bottles until the first pupae were observed in any bottle of a set. Eclosing offspring were collected and removed everyday for the first 5 days and then once every 3-4 days until there were dark pupae observed in the control bottles, which served as a criterion for determining the end of 1^{st} generation and beginning of 2^{nd} generation.

For each inbred line we calculated stress level and the number of lethal equivalents under benign laboratory conditions and stressful field conditions (β_{benign} and β_{stres}) , using the average outbred productivity. We used the average outbred survival under lab and field conditions to calculate stress level and average outbred survival per set to calculate lethal equivalents. We then analyzed the effects of ENV (lab, field summer, field winter), YEAR (1-3, each consisting of 1 summer and 1 winter season) and LINE (6 inbred lines) on the number of lethal equivalents using an ANOVA (SAS® version 9.1). ENV and YEAR were fixed effects while LINE was random. Interactions with a p value greater than 0.25 were removed and the analysis was re-run (Kirk, 1982). We used linear regression to test the hypothesis that the difference between inbreeding depression under field and lab conditions ($\beta_{stress} - \beta_{benign}$) is greatest during the most stressful months of the year. Finally, we compared stress levels

experienced during the winter and summer seasons (SEASON) in the field using and ANOVA.

To compare our results to that of Fox and Reed (2010) we performed both unweighted and weighted linear regression on our data set for *Drosophila* and that of the 27 species included in the Fox and Reed (2010) meta-analysis (data supplied in Table S4). The difference in lethal equivalents in stressed and benign environments ($\beta_{stress} - \beta_{benign}$) was weighted by the reciprocal of the number of parameter estimates per study (i.e. estimates of inbreeding depression). We then compared the two slopes of the relationship between stress level and ($\beta_{stress} - \beta_{benign}$) from our *Drosophila* data set and that of Fox and Reed (2010) using ANCOVA.

Supplementary Materials

Supplementary Table 1.1: Summary of studies used in the analysis of inbreeding depression and stress level in laboratory populations of *Drosophila*.

STUDY	SPECIES	STRESS TYPE	β_{stress}	β_{benign}	Stress Level
Enders and Nunney (in prep)	*D. melanogaster*	Biotic	1.80	0.34	0.45
Enders and Nunney (in prep)	*D. melanogaster*	Biotic	1.23	0.34	0.47
Enders and Nunney 2010	*D. melanogaster*	Biotic	1.76	0.85	0.52
Ferriera and Amos 2006	*D. melanogaster*	Biotic	0.73	0.37	0.33
Fowler and Whitlock 2002*	*D. melanogaster*	Biotic	0.33	0.33	0.15
Enders and Nunney (in prep).	*D. melanogaster*	Chemical	0.63	0.34	0.45
Ferriera and Amos 2006	*D. melanogaster*	Chemical	0.73	0.37	0.20
Ferriera and Amos 2006	*D. melanogaster*	Chemical	0.84	0.37	0.18
Kristensen et al 2003*	*D. buzzati*	Chemical	0.21	0.33	0.18
Miller 1994	*D. melanogaster*	Chemical	1.16	1.14	0.27
Kristensen et al 2008*	*D. melanogaster*	Cold	1.24	0.10	0.31
Fowler and Whitlock 2002*	*D. melanogaster*	Cold	0.41	0.33	0.03
Enders and Nunney (in prep)	*D. melanogaster*	Heat	1.23	0.34	0.28
Bijlsma et al 1999	*D. melanogaster*	Heat	1.99	1.11	0.76
Dahlgaard n Hoffmann 2000	*D. buzzati*	Heat	1.59	0.33	0.58
Kristensen et al 2003*	*D. melanogaster*	Heat	0.93	0.10	0.38
Kristensen et al 2008*	*D. melanogaster*	Heat	1.26	0.56	0.68
Mikkelson et al 2010	*D. melanogaster*	Heat	2.39	0.29	0.68
Mikkelson et al 2010	*D. melanogaster*	Heat	0.62	0.65	0.35
Dahlgaard n Loeschke 1997*	*D. buzzati*	Heat	0.72	0.33	0.22
Kristensen et al 2003*	*D. melanogaster*	Heat	0.92	1.14	0.18
Miller 1994	*D. melanogaster*	Heat	1.04	1.14	0.09

* data points shared with Fox and Reed (2010)

Supplementary Table 1.2: Summary of random simulations of stress level and inbreeding depression. A) Formulae for parameters used in the simulations B) Results of 10 random simulations using only cases were inbreeding depression was greater under stress ($\beta_{stress} > \beta_{benign}$).

A)

	Fitness		**Stress_Level**	**Lethal Equiv(B)**
	Outbred	*Inbreed*	1- ($W_{OB\text{-}S}/W_{OB\text{-}B}$)	-(1/F)ln(W_{OB}/W_{OB})
Benign	**$W_{OB\text{-}B}$**	**$W_{IB\text{-}B}$**		
	1	u(1,1/2)		
Stress	**$W_{OB\text{-}S}$**	**$W_{IB\text{-}S}$**		
	u(1,1/4)	u($W_{OB\text{-}S}$, 0)		

B)

	Slope	**Upper 95% CI**	**Lower 95% CI**	**p value**
Level F	0.3490	-0.0661	0.7641	0.0994
0.25	-0.1824	-0.6067	0.2420	0.3996
	0.1015	-0.3151	0.5181	0.6331
	-0.1410	-0.5596	0.2777	0.5093
	0.2903	-0.1229	0.7036	0.1685
	-0.0084	-0.1122	0.0955	0.8745
1.00	0.0062	-0.0968	0.1092	0.9066
	0.1036	-0.0013	0.2085	0.0530
	-0.0408	-0.1444	0.0628	0.4399
	-0.0048	-0.1100	0.1004	0.9290

Chapter 2

The effect of the nature and timing of exposure to environmental stress on the magnitude of inbreeding depression in *Drosophila melanogaster*

Abstract

The potential for environmental stress to exacerbate the deleterious effects of inbreeding is a major concern in conservation biology. However, it is unclear what factors contribute to the wide variation in the expression of inbreeding depression under stress previously observed. We aimed to determine how the nature and timing of exposure to stress affect inbreeding depression using *Drosophila melanogster*. Inbred and outbred larva were exposed to biotic stressors (increased larval competition, *Serratia marsescens*), abiotic stressors (8.2% ETOH, 33/28C alternating high temperature) and standard laboratory conditions during development. Inbreeding depression was measured during the stressful period (larval to adult survival) and post exposure to stress (male mating success). Heat and larval competitive stress significantly amplified inbreeding depression for larval survival, while ethanol and bacteria did not, possibly because more genes and hence a greater number of deleterious mutations may be expressed under heat and competitive stress. In addition, we found that the biotic stresses reduced inbreeding depression in male mating success, presumably via purging less fit larvae, suggesting a correlation between fitness of larvae exposed to biotic stresses and male mating success.

Introduction

Natural populations face a continuous onslaught of stresses including pollution, climatic change, and disease, and as a result stress is recognized has having major implications for both short-term survival and long-term adaptation (Parsons 1996; Loeschcke et al. 2004; Frankham 2005). When taken to extremes, environmental stress can threaten the survival of a population, but more generally the effect of stress is to significantly reduce the fitness of the individual in a population relative to more benign conditions (Hoffmann and Parsons 1991; Imasheva and Loeschcke 2004; Bijlsma and Loeschcke 2005). Using this approach the level of stress can be defined as the reduction in fitness due to some environmental challenge (Fox and Reed 2010).

The deleterious effects of stress are often amplified in inbred individuals (Crnokrak and Roff 1999; Armbruster and Reed 2005), rendering small inbred populations particularly vulnerable to stressful conditions (Bijlsma et al. 2000; Reed et al. 2002). Individuals may be inbred because their parents are close relatives or because they are members of a small population where all individuals share a high degree of coancestry, since inbreeding can be defined as the probability that two gene copies chosen at random are identical by descent (IBD) and is measured by the inbreeding coefficient (F) (Wright 1931). In either case, increased IBD due to inbreeding increases the expression of recessive deleterious alleles, which can lead to a reduction of fitness known as inbreeding depression (Charlesworth and Willis 2009; Kristensen et al. 2010). Environmental

stress is predicted to exacerbate this problem by increasing the number of deleterious alleles expressed and/or amplifying the negative effects these alleles have on fitness (Bijlsma et al. 1999; Dahlgaard and Hoffmann 2000; Haag et al. 2002; Reed et al. 2003). However, there appears to be no consistent or predictable effects of environmental stress on the expression of inbreeding depression (Keller and Waller 2002; Armbruster and Reed 2005; Waller et al. 2008)

In *Drosophila,* the number of studies that report a significant increase in the level of inbreeding depression under stress (Miller 1994; Bijlsma et al. 1999; Bijlsma et al. 2000; Dahlgaard and Hoffmann 2000; Reed et al. 2002) are comparable to those that do not (Hedrick 1994; Dahlgaard et al. 1995; Fowler and Whitlock 2002; Kristensen et al. 2003). Even within the same study, inbreeding depression has been shown to respond positively, negatively, or not at all to alternate sources of stress in *Drosophila* (Bijlsma et al. 1999; Dahlgaard and Hoffmann 2000). The unpredictable effects of stress on inbreeding depression suggest a need to explore additional factors that may influence the relationship between inbreeding and stressful conditions. The overall level of mortality that a stress induces (stress level) has been shown to be an important factor determining the expression of inbreeding depression (Fox and Reed 2010). A recent meta-analysis of 27 species of vertebrates, invertebrates, and plants found that inbreeding depression scales linearly with stress levels (Fox and Reed 2010). In *Drosophila*, Enders and Nunney (in prep) also found a strong

positive relationship between stress level and the magnitude of inbreeding depression for both laboratory populations and those experiencing fluctuating stress levels in the field. However, both meta-analyses found that stress level alone explains approximately 50% of the variance in inbreeding depression expressed under stressful conditions. These results suggest additional factors may affect the expression of inbreeding depression under stress, for example: 1) the type of stress and 2) the timing of exposure to the stress (e.g. one vs. all life history stages).

Fundamental differences in the way in which stressors affect the expression of genes and hence the expression of genetic load may translate into qualitatively different outcomes for the survival of inbred individuals. For example, chemical stressors are typically thought to involve a few specific loci (Roush and McKenzie 1987; Hoffmann and Parsons 1991), so they may usually amplify inbreeding depression less than other types of stress that affect a broader spectrum of the genome. Only in those rare cases where deleterious alleles were present in the specific genes or pathways directly involved in processing of or the physiological response to certain chemicals, would inbreeding depression expected to be high. Such environment-specific effects on the overall number and specificity of deleterious alleles expressed predict that stress type may be a primary determinant of the magnitude of inbreeding depression. However, only a few studies have used more than one type of stress (Miller 1994; Bijlsma et al. 1999; Bijlsma et al. 2000; Reed and Bryant

2001; Fowler and Whitlock 2002; Kristensen et al. 2003; Waller et al. 2008) and the majority focus on abiotic stresses such as heat and chemical stress (see review by Armbruster and Reed 2005). In addition, the lack of standardization of stress level within or across studies prevents a direct comparison of the effects of different stress types on the expression of inbreeding depression. As a result, variation in the way in which different stress types interact with the deleterious consequences of inbreeding is largely unexplored.

Variation in levels of inbreeding depression under different types of stress may also depend on the timing of exposure to stress, or the developmental period at which individuals experience stress. One widely held view is that stress acts in general to increase inbreeding depression across all life history traits regardless of the time of exposure (Hoffmann and Parsons 1991). This hypothesis assumes that development under stress can result in an individual that is not at its physiological optimum and predicts stress to have long lasting phenotypic effects on an individual that will lead to enhanced inbreeding depression both during development and across all subsequent life history stages. However in populations that are not fully inbred, if stress increases selection against deleterious mutations that affect fitness at multiple life-history stages, environmental stress applied during development could purge genetic load and reduce the magnitude of inbreeding depression in later life history stages. Consistent with this purging hypothesis, it has been shown in *D. melanogaster* that larval competitive stress significantly amplifies inbreeding

depression during the stressed larval period (larval to adult survival), but no such increase occurred in the post-stress reproductive traits of female fecundity and male mating success (Enders and Nunney 2010). However, the absence of a post-stress reduction in inbreeding depression left the interpretation ambiguous. Furthermore, it remains to be seen if this pattern is a general one, i.e. do other stresses lower (or at least fail to increase) inbreeding depression in later life history stages.

The aim of this study was to evaluate how inbreeding depression is affected by both stress type and timing of exposure. We endeavored to control the stress level by using conditions that increase the mortality of outbred individuals by a constant amount. Using these standardized conditions this study addressed two main questions: 1) Do different types of stress cause different levels of inbreeding depression both during and after exposure? and 2) Does exposure to stress during development reduce inbreeding depression in later life history stages? We measured inbreeding depression for larval survival and male mating success (post stress) under four types of environmental stress (2 abiotic and 2 biotic) and control conditions using populations of *D. melanogaster* ranging in level of inbreeding (F =0, 0.25 and 0.50). This is the first study to incorporate multiple abiotic and biotic stresses while controlling the baseline level of mortality induced by each stress.

Methods

Base Population and Inbreeding Design

In October 2008 outbred stock populations of *D. melanogaster* were collected from two locations in Northern California, the Galante Winery in Carmel Valley (Gala) and the Mayo Family Winery in Sonoma Valley (Mayo). In order to minimize any modification of the genetic architecture through selection or inbreeding, 400 pairs of wild caught flies from each location were placed in vials and reared in the laboratory at 18C. Their progeny were outcrossed by taking a single male and female from each of the 400 pairs (per population) and mating them in a circular design, whereby each male is mated to the female from the next vial. Each of the two outbred stock populations (Gala and Mayo) were reared at 18C using the above circular outcrossing design for 6 generations prior to the start of the experiment.

From each of the large outbred stock populations described above, 10 inbred lines were created for each of two levels of inbreeding (F= 0.25, 0.5) using the appropriate number of generations of full-sib mating. To create each inbred line (10 per stock population per level of F), 5 replicate full-sib pairs were set up in separate vials each generation. The offspring of one of the five pairs, chosen at random, were used to establish the next generation of sibling mating (5 new sibling pairs). This method of sibling mating was done for three generations to reach an F = 0.50 and for one generation to reach an F = 0.25. The level of inbreeding (F) was calculated according to the equation for full-sib mating: F_t=

$(1+ F_{t-1} - F_{t-2})/4$ (Falconer and Mackay 1996). Each of the two large outbred stock populations (F = 0) were maintained simultaneously as 400 pairs of individuals, using the circular mating design described above. All of the above inbred and outbred lines were reared at 25C.

All inbred lines and the outbred base population were synchronized so that individuals reached the desired level of inbreeding (F= 0, 0.25 or 0.50) at the same time. When the inbred lines had reached the desired level of F they were expanded to large inbred bottle populations (N=100) in order to rear enough adults for use in the experiment. This was done by taking 100 virgin progeny (50 males: 50 females) from a single sib pair at the final generation of sib mating and placing them into a large bottle to randomly mate. After one week the adults were transferred to a second bottle of new food. The progeny of these two bottles were then used to create two replicate experimental bottle populations (N=100, 50:50 virgin males and females) for each of the inbred lines (F= 0.25 ad 0.50). Five replicate outbred bottle populations (N=100) were created from each of the two stock populations by combining one virgin male and female from each of fifty randomly chosen pairs from the 400 stock pairs used to maintain the outbred populations. The offspring (F1) of the inbred and outbred bottle populations were used in the experimental treatments described below. The experiment was replicated twice (Sets I and II) with both stock populations (Gala and Mayo).

Environmental Stress Treatments

The F1 larvae were subject to four types of stress plus control conditions in vials containing 10 ml of the appropriate food medium. Each condition was replicated three times per line. The control vials contained a standard food medium consisting of molasses, cornmeal, yeast, water and the antifungal agent Tegosept. The stress treatments were: *Abiotic:* a) Heat Stress: Larvae were reared under fluctuating high temperatures, 28C for 12 hours and 34C for 12 hours. b) Chemical Stress: Larvae were reared on food containing 8.2% ETOH. *Biotic:* c) Bacterial Stress: Larvae were reared on standard food to which 4 drops of the pathogenic bacteria *Serratia marcescens* were added (~$4.8x10^{10}$ CFU/vial). d) Intraspecific Competitive Stress: Larvae were reared on a 1/3x concentration of food. This was done by adding 2/3 agar (18g/L) to 1/3 of the standard 1x food medium. The stress treatments were chosen based on preliminary experiments demonstrating an average 45-50% reduction in survival relative to control conditions. Therefore the level of stress was standardized (baseline mortality) across all four stress treatments.

To set up the experimental vials, each inbred and outbred bottle population described above was transferred to an empty glass bottle capped with a petri dish containing standard food medium and allowed to lay eggs for a period of 8 hours. First instar larvae were collected in groups of 100 larvae for up to 8 hours from the laying dish. This ensured that all larvae were at ± 4 hours apart in development. For each experimental vial, larvae were transferred using a

paintbrush to vials containing 10ml of food medium in the ratio of 100 experimental larvae to 150 of a standard competitor *spa* (*D.mel* laboratory stock with recessive *spa* eye mutation). All vials were maintained at 25C except for the heat stressed vials.

Fitness Measures

Larval to adult survival was measured under the four stress and the benign control conditions. Males that survived these treatments were used to measure mating success (see below).

1) Larval to adult Survival

Following the set up of first instar larva in the experimental vials (day 1), eclosing adults were observed on days 9-10 at 25C or on days 8-9 at 33/28C. All emerging adults were counted and removed every 3-4 days until approximately day 20-21 at 25C and day 19-20 at 33/28C, by which time the number of first generation progeny emerging per vial had typically diminished to zero (or nearly so) over the final 3-4 day counting interval (day ~19-21 or ~18-20 respectively for temperature regimes) and a large number of dark pupae representing the next generation were observed. There was always a clear distinction between the first and second generation.

Larval-adult survival was measured in two ways: as the proportion of test larvae surviving to eclosion (LS) and as the larval competitive index (LCI), which is the proportion that eclosed per vial of the test line relative to the proportion of *spa* competitors (Knight and Roberston 1957).

2) Male Mating Success

Adult males that emerged from the larval-adult survival assay were used to measure male mating success using methods described in Enders and Nunney (2010). In summary, five virgin males (full sibs, 5-8 days old) were randomly selected from each vial and placed with 15 unrelated virgin competitor *spa* males and 10 unrelated virgin *spa* females in new vials containing 10ml food for two hours at 25°C. Females were removed using light anesthesia and transferred individually to new vials. After ~2 weeks the progeny of each *spa* female were scored for eye color to determine her mate (100% wild type if mated to a test male).

Male mating success was measured both as a proportion of test males mating (MS) and, relative to the standard competitor *spa*, as the male competitive index (MCI), which was defined as the proportion of females inseminated by test males divided by the proportion of females inseminated by the standard competitor *spa* males.

Data Analyses

Inbreeding depression was analyzed for larval survival and male mating success using the competitive indices (LCI or MCI). The competitive indices were log transformed and used to calculate the number of lethal equivalents (β), a measure commonly used to compare the effects of inbreeding on fitness across studies, species/taxa, and environments (Hedrick and Kalinowski 2000; Armbruster and Reed 2005). The number of lethal equivalents per haploid

genome is calculated as the slope of the regression of ln(fitness) versus F, where F is the level of inbreeding (F = 0, 0.25 or 0.50) and fitness was either LCI or MCI (Morton et al. 1956). We calculated the number of lethal equivalents for two population across five environmental treatments in two replicate sets of the experiment (20 total values). To determine whether the number of lethal equivalents differed under benign and stressful conditions an ANOVA (SAS Version 9.1 for Windows ®) was run using the following variables: ENV (Control, Heat, Ethanol, Bacteria, Competition), POP (Gala,Mayo), and SET (Set I,Set II). ENV was fixed while SET and POP were random variables. The following a priori planned comparisons of lethal equivalents were performed: 1) Benign versus Stress (4 stresses grouped) 2) Abiotic verses Biotic Stresses 3) Ethanol verses Heat and 4) Bacteria verses Competition. Post hoc multiple comparisons were calculated using a Tukey test with Games and Howell's correction for unequal variances to determine whether inbreeding depression differed across the environmental treatments.

To determine whether purging of genetic load occurred during the larval period, levels of inbreeding depression (β) for larval survival (LS) and male mating success (MCI) were correlated. This was done for each population (Gala and Mayo), set (Set I and II), and environmental conditions separately. Larval survival (LS) was used in this analysis instead of LCI, since LS is a direct measure of the potential purging. An ANCOVA was also run to determine whether inbreeding depression post exposure to stress (male mating success)

was affected by inbreeding depression during the larval period (B_LS: lethal equivalents larval survival), category of stress (S_TYPE: abiotic vs biotic) and the specific stress nested within S_TYPE (STRESS: Heat, Ethanol, Bacteria, Competition). Non-significant interactions ($p > 0.25$) were removed from the above models and the analysis was rerun (Kirk 1982).

Results

Inbreeding Depression During Exposure to Stress

In general, development under stressful conditions amplified inbreeding depression for larval to adult survival ($p < 0.01$, Table 2.2.1), with the average number of lethal equivalents expressed for larval survival (LCI) three fold higher under stressful conditions ($\beta_{avg} = 1.47 \pm 0.18$) relative to benign control conditions ($\beta_{avg} = 0.46 \pm 0.01$). This corresponds to an average 25% ($F= 0.25$) and 39% ($F= 0.50$) reduction in inbred larval survival relative to outbred larva under stress compared to a 14% ($F= 0.25$) and 16% ($F= 0.50$) relative reduction in survival under benign conditions (Table 2.2). The level of inbreeding depression induced by the four stress treatments did not significantly differ from each other (Figure 2.2.1a). Abiotic and biotic stresses caused similar levels of inbreeding depression ($F_{1,15}= 1.84$ $p >0.05$) as did ethanol and heat stress ($F_{1,15}= 3.52$ $p >0.05$). However, heat and larval competitive stress significantly increased inbreeding depression relative to the benign control conditions (Table 2.2.1a and Figure 2.2.1a).

Figure 2.2 shows the relationship between stress level and inbreeding depression for larval survival for each population, set, and stress. We found a significant positive relationship between stress level and increase in number of lethal equivalents under stress ($B = 1.94 \pm 0.41$, $p < 0.001$).

Inbreeding Depression Post Exposure to Stress

Males surviving exposure to stressful conditions during development had reduced inbreeding depression for mating success compared to males that experienced benign conditions ($F_{1,15}= 7.45$ $p = 0.02$, Figure 2.2.1b). We found that the number of lethal equivalents for competitive mating success (MCI) expressed in males exposed to stressful conditions ($\beta_{avg} =1.60 \pm 0.14$) was on average 30% lower than males reared under benign conditions ($\beta_{avg} = 2.32 \pm 0.23$). In terms of percent matings, inbred males exposed to stress had on average 21% (F= 0.25) and 32% (F= 0.50) reduced mating success relative to outbred males whereas inbred males that experienced benign conditions showed a 45% (F= 0.25) and 50% (F= 0.50) relative reduction in fitness (Table 2.3). We did not find significant differences in the number of lethal equivalents expressed in males exposed to abiotic versus biotic stress ($F_{1,15}= 1.74$ $p >0.05$) or between the two abiotic (heat and ethanol) or two biotic stresses (bacteria and competition) ($F_{1,15}= 0.006$ $p >0.05$ and $F_{1,15}= 1.38$ $p >0.05$ respectively). However, the two biotic stresses caused a significant reduction in the level of inbreeding depression for male mating (Table 2.2.1b and Figure 2.2.1b).

We investigated whether greater levels of inbreeding depression during

the stressed period (larval survival) correlated with lower levels of inbreeding depression post exposure (male mating success), indicating purging of genetic load (Figure 2.2). Inbreeding depression for male mating was negatively correlated with inbreeding depression for larval survival only in the biotic stresses (for larval competition B = -0.75 ± 0.25, $p_{1\text{-tailed}} = 0.05$; for bacterial stress B = -0.40 ± 0.07, $p_{1\text{-tailed}} = 0.02$), wile it was non-significant and positive slope for both abiotic stresses (Table 2.4 and Figure 2.3).

Discussion

The Effect of Stress Type on Inbreeding Depression

Although environmental stress is widely recognized as amplifying inbreeding depression, the considerable variation in observed effects of stress on inbreeding depression suggests additional factors need to be considered (Armbruster and Reed 2005; Waller et al. 2008). The results of this study show that the nature or type of stress that individuals are exposed to can be an important determinant of the magnitude of inbreeding depression. Standardization of stress level enabled a direct comparison of the effect of different types of stress on levels of inbreeding depression expressed both during and after exposure. While there is no distinction between abiotic and biotic forms of stress, we find that the highest inbreeding depression was induced by heat and competitive stress compared to ethanol and bacterial stress (Figure 2.2.1a).

Our results have important implications for understanding the genetic basis of inbreeding depression. It is has been proposed that inbreeding depression is amplified by increasing the number of deleterious alleles expressed and/or by increasing the strength of selection against deleterious alleles (Falconer and Mackay 1996; Hedrick and Kalinowski 2000). Therefore, those environmental stresses affecting a broader spectrum of the genome are therefore predicted to induce greater reductions in fitness upon inbreeding. For example, heat may be a more generalized stress with consequences across a broad range of biological pathways whereas chemical stress may affect far fewer pathways (Hoffmann and Parsons 1991), which is consistent with our finding of higher inbreeding depression under heat stress relative to ethanol stress. In addition, the number of genes exhibiting altered expression patterns relative to benign conditions has been shown to vary considerably across different types of stress in *Drosophila.* Both heat stress and starvation have been shown to induce significant changes in expression levels in several thousand genes (Harbison et al. 2005; Kristensen et al. 2005; Sorensen et al. 2005). In contrast, different types of chemical and oxidative stress have caused changes in relatively few genes (Girardot et al. 2004; Landis et al. 2004). Results from the current study showed the greatest cost to being inbred occurred under larval competitive stress and fluctuating high temperature (Figure 2.2.1a), suggesting a greater number of genes or biological pathways may be affected or involved in the interaction between these stresses.

In general, little is known about the molecular and biochemical underpinnings of inbreeding depression (Kristensen et al. 2010; Ouborg et al. 2010), and in particular the genetic basis of inbreeding under environmental stress is unknown. Recent work in *Drosophila* suggests that inbreeding leaves a detectable molecular fingerprint (Kristensen et al. 2010), including the up-regulation of heat shock proteins and other major stress response and metabolic pathways (Kristensen et al. 2002; Kristensen et al. 2005; Pedersen et al. 2005; Kristensen et al. 2006). Currently, only one study has investigated the interaction between inbreeding and stress on gene expression (Kristensen et al. 2006). Further work is needed to determine if variation in levels of inbreeding depression under stress can be explained by differences in the number of genes or pathways affected by the type of environmental stressor.

Despite our efforts to equalize the level of stress across all four types of stress, our heat treatment was found to be less stressful (stress level = 0.28) than the other three stresses (avg stress level = 0.45). However, despite causing the lowest mortality in outbred individuals, heat stress caused among the highest levels of inbreeding depression for larval survival (Figure 2.2.1a). This suggests heat may have a greater effect on the physiology of inbred organisms and/or involve a greater number of deleterious alleles being expressed than other forms of biotic and abiotic stress. Future work incorporating multiple sources of stress should standardize the stress level, especially if heat stress is involved, in order to properly interpret results involving interactions with inbreeding.

The Effect of the Timing of Exposure to Stress

In general, it is unclear how experiencing stressful conditions during development will affect the expression of inbreeding depression across multiple life history stages (Waller et al. 2008). Stress could act to amplify inbreeding depression across the entire life cycle of an organism, but could also reduce inbreeding depression in later life history stages due to greater selection against individuals expressing deleterious recessive alleles. Our results support the purging hypothesis, whereby stressed larval development resulted in lowered inbreeding depression in post exposure male mating success (Figure 2.2.1b). However, this reduction was only significant for the two biotic stresses, increased larval competition and exposure to the pathogenic bacterium *Serratia marsescens* (Table 2.2.1b and Figure 2.2.1b). Purging is predicted to occur if fitness for larval survival and mating success are correlated because some genes are important for both larval survival and male mating.

In order to further investigate potential differences in the ability of different stressors to purge genetic load, we examined the effect of inbreeding depression during larval competition on inbreeding depression for male mating success (Figure 2.2). We found that only under biotic stress was inbreeding depression for male mating success and larval survival significantly negatively correlated, indicating purging of deleterious alleles during larval development (Figure 2.3). These results suggest the biotic stressors affected genes important for both larval development and male mating ability, i.e. selection against recessive

deleterious mutations during the stressed larval stage results in improved mating success due to fewer males expressing these mutations as inbred adults. The abiotic stresses showed a non-significant positive relationship between inbreeding depression during and after exposure (Figure 2.3). This suggests that these stresses may have persistent effects across multiple life history stages; however even these stresses showed a non-significant decrese in inbreeding depression for male mating success. Future challenges include identifying those stresses that effectively purge genetic load and elucidating the mechanisms by which this can occur (Agrawal and Whitlock 2010).

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Tables and Figures

Table 2.1: ANOVA comparing inbreeding depression under benign and stressful conditions for A) larval survival (LCI) and B) male mating success (MCI). The analysis compares inbreeding depression (lethal equivalents) under control conditions and the four stress treatments (ENV). Two populations of *D. melanogaster* (POP: Gala and Mayo) were used and the experiment was replicated twice (SET). Interactions were non-significant ($p > 0.25$) and removed from the models.

A) Larval Survival

Source	*DF*	*MS*	*F*	*p value*
ENV	4	1.644	6.09	**0.005**
Stress vs Control	1		10.97	**0.005**
Biotic vs Abiotic	1		1.84	0.195
Ethanol vs Heat	1		3.51	0.080
Larval Competition vs Bacteria	1		2.13	0.120
POP	1	0.776	2.88	0.114
SET	1	0.188	0.70	0.420
Error	13	0.270		

B) Male Mating Success

Source	*DF*	*MS*	*F*	*p value*
ENV	4	0.755	5.43	**0.017**
Stress vs Control	1		7.45	**0.016**
Biotic vs Abiotic	1		1.74	0.207
Ethanol vs Heat	1		0.01	0.940
Larval Competition vs Bacteria	1		1.38	0.258
POP	1	0.003	0.02	0.902
SET	1	0.573	1.72	0.222
Error	9	0.139		

Table 2.2: Summary of the average (± se) larval survival under benign and stressful conditions. Larval-adult survival was measured both relative to a standard competitor (LCI) and in absolute terms as the percent survival. Populations were bred by sibmating or outcrossing to reach three levels of inbreeding (F) replicated across two original stock popuations (Gala and Mayo). From these populations larval survival was measured under benign control conditions and four stress treatments (Heat, Ethanol, Bacteria, and Competition). N is the number of lines for each level of inbreeding.

F		Larval to Adult Survival				
		CONTROL	HEAT	ETOH	BACTERIA	COMP
0	GALA					
	LCI	1.66 ± (0.04)	4.23 ± (0.14)	1.44 ± (0.05)	1.21 ± (0.03)	2.01 ± (0.15)
	% Surv	86.68 ± (0.86)	66.96 ± (1.35)	49.69 ± (2.24)	45.62 ± (2.02)	47.38 ± (1.71)
		N = 10	N = 10	N = 10	N = 10	N = 10
	MAYO					
	LCI	1.63 ± (0.03)	3.46 ± (0.16)	1.44 ± (0.05)	1.20 ± (0.03)	1.55 ± (0.11)
	% Surv	86.48 ± (1.01)	56.88 ± (2.23)	44.88 ± (2.26)	45.85 ± (1.78)	47.81 ± (2.90)
		N = 10	N = 10	N = 10	N = 10	N = 10
0.25	GALA					
	LCI	1.34 ± (0.03)	2.81 ± (0.12)	1.07 ± (0.07)	0.91 ± (0.04)	1.21 ± (0.09)
	% Surv	74.42 ± (1.51)	49.09 ± (1.58)	34.42 ± (2.60)	34.33 ± (2.08)	33.20 ± (2.16)
		N = 19	N = 18	N = 19	N = 18	N = 13
	MAYO					
	LCI	1.34 ± (0.02)	2.56 ± (0.08)	1.08 ± (0.04)	0.88 ± (0.04)	1.13 ± (0.11)
	% Surv	75.74 ± (0.91)	50.68 ± (1.28)	40.27 ± (1.71)	34.86 ± (2.21)	31.10 ± (2.18)
		N = 19	N = 14	N = 16	N = 16	N = 13
0.50	GALA					
	LCI	1.29 ± (0.03)	1.59 ± (0.10)	1.02 ± (0.06)	0.62 ± (0.05)	0.77 ± (0.08)
	% Surv	72.67 ± (1.51)	33.02 ± (1.79)	36.60 ± (2.44)	24.85 ± (2.65)	22.58 ± (1.94)
		N = 17	N = 16	N = 19	N = 15	N = 13
	MAYO					
	LCI	1.28 ± (0.02)	2.11 ± (0.11)	0.97 ± (0.05)	0.79 ± (0.04)	0.66 ± (0.07)
	% Surv	72.74 ± (1.25)	41.11 ± (1.95)	36.20 ± (2.42)	31.88 ± (2.07)	21.62 ± (2.14)
		N = 16	N = 16	N = 16	N = 15	N = 13

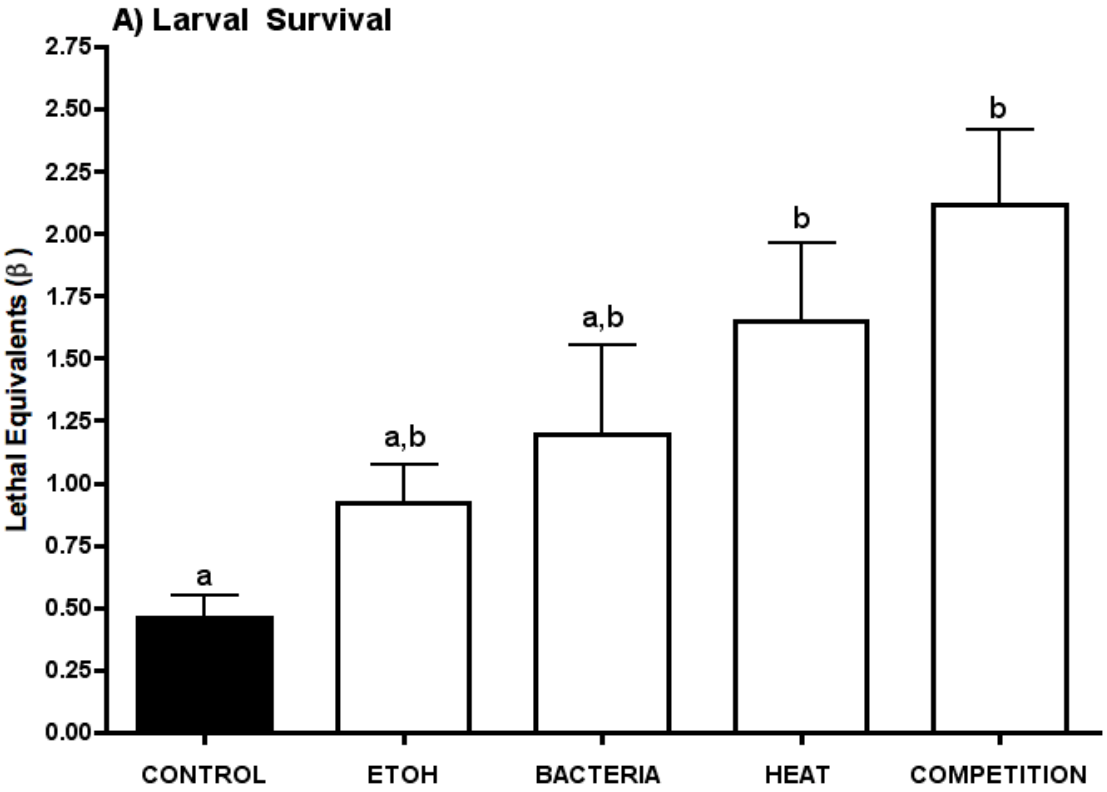
Table 2.3: Summary of the average (± se) male mating success under benign and stressful conditions. Mating success was both measured relative to a standard competitor (MCI) and in absolute terms as the percent females mated. For additional detail see Table 2.2.

		Male Mating Success				
F		**CONTROL**	**HEAT**	**ETOH**	**BACTERIA**	**COMPETITION**
0	**Set I**					
	MCI	3.54 ± (0.37)	0.73 ± (0.07)	2.41 ± (0.37)	1.34 ± (0.20)	1.27 ± (0.12)
	% Matings	0.74 ± (0.02)	0.40 ± (0.02)	0.63 ± (0.03)	0.51 ± (0.04)	0.53 ± (0.02)
		N = 10	N = 10	N = 10	N = 10	N = 10
	Set II					
	MCI	2.83 ± (0.33)	0.62 ± (0.05)	1.74 ± (0.20)	1.36 ± (0.19)	1.45 ± (0.16)
	% Matings	0.69 ± (0.02)	0.36 ± (0.02)	0.59 ± (0.03)	0.53 ± (0.03)	0.56 ± (0.03)
		N = 10	N = 10	N = 10	N = 10	N = 10
0.25	**Set I**					
	MCI	1.39 ± (0.15)	0.51 ± (0.04)	1.09 ± (0.13)	0.71 ± (0.09)	0.89 ± (0.09)
	% Matings	0.53 ± (0.02)	0.32 ± (0.02)	0.47 ± (0.02)	0.38 ± (0.03)	0.44 ± (0.02)
		N = 19	N = 18	N = 19	N = 18	N = 13
	Set II					
	MCI	0.99 ± (0.06)	0.30 ± (0.03)	1.04 ± (0.11)	1.10 ± (0.10)	1.00 ± (0.09)
	% Matings	0.48 ± (0.02)	0.21 ± (0.01)	0.47 ± (0.02)	0.49 ± (0.02)	0.48 ± (0.02)
		N = 19	N = 14	N = 16	N = 16	N = 13
0.50	**Set I**					
	MCI	1.07 ± (0.12)	0.43 ± (0.06)	0.76 ± (0.10)	0.70 ± (0.08)	0.54 ± (0.06)
	% Matings	0.47 ± (0.02)	0.27 ± (0.02)	0.38 ± (0.03)	0.38 ± (0.03)	0.32 ± (0.03)
		N = 17	N = 16	N = 19	N = 15	N = 13
	Set II					
	MCI	0.86 ± (0.08)	0.19 ± (0.01)	1.04 ± (0.13)	0.96 ± (0.19)	0.85 ± (0.09)
	% Matings	0.43 ± (0.02)	0.16 ± (0.01)	0.47 ± (0.02)	0.43 ± (0.03)	0.43 ± (0.03)
		N = 16	N = 16	N = 16	N = 15	N = 13

Table 2.4: ANCOVA comparing the level of inbreeding depression (lethal equivalents) for male mating success (MCI) after exposure to stressful conditions. The analysis includes STRESS: abiotic stress (heat and ethanol) and biotic stress (bacteria and larval competition), and the level of inbreeding depression expressed in larval survival during exposure to stress (ID_LS) as a covariate. The analysis was split by type of stress (abiotic vs biotic) due to a significant interaction between these categories and ID_LS ($F_{1,8}$ = 9.4 p = 0.015). Non-significant interactions with the covariate were removed and the model was rerun.

Source	*DF*	*MS*	*F*	*p value*
Abiotic Stresses				
ID_LS	1	1.081	3.880	0.120
Slope = 0.50 ± 0.40				
STRESS	1	0.315	1.130	0.348
Error	4	0.278		
Biotic Stresses				
ID_LS	1	0.665	**7.870**	**0.038**
Slope = -0.27 ± 0.23				
STRESS	1	0.879	**10.400**	**0.023**
Error	4	0.278		

Figure 2.1: Inbreeding depression for larval survival and male mating success under benign and stressful conditions. The number of haploid lethal equivalents (β) was measured relative to a standard competitor using (A) the larval competitive index (LCI) or (B) the male competitive index (MCI). The number of lethal equivalents for the control conditions and each individual stress treatment are shown. Lower case letters correspond to treatments where inbreeding depression (β) is significantly different ($p < 0.05$).


A) Larval Survival
Lethal Equivalents (β)
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
a,b
a,b
b
b
CONTROL
ETOH
BACTERIA
HEAT
COMPETITION

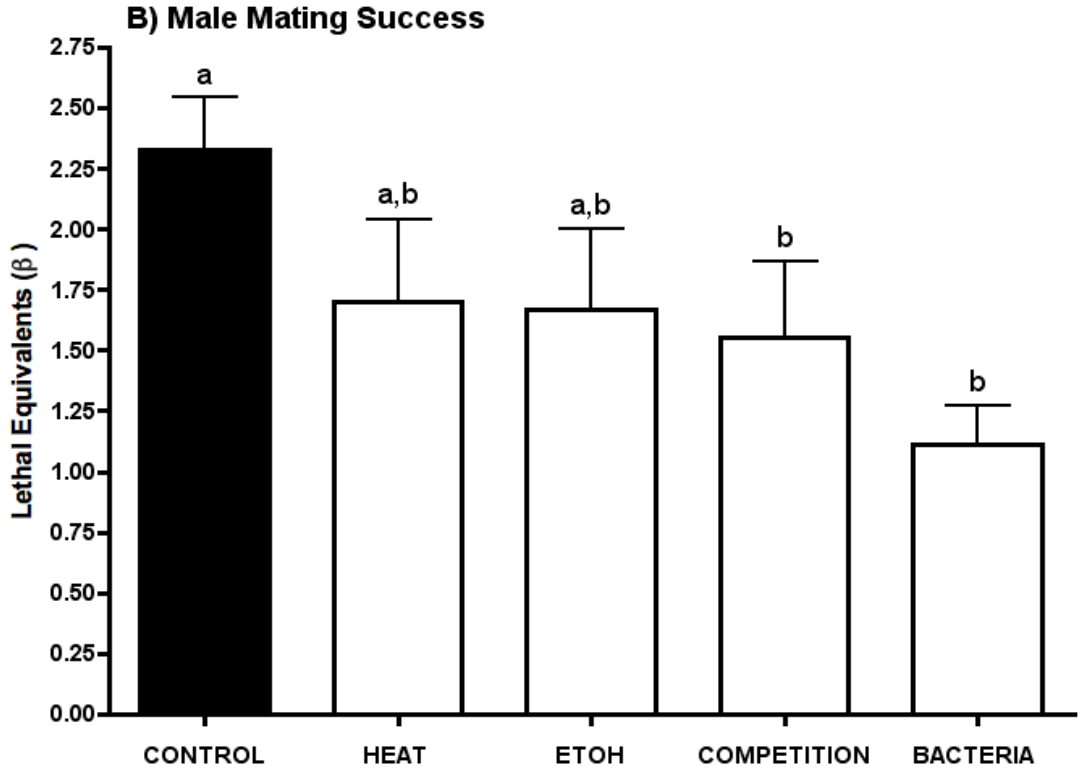

B) Male Mating Success
Lethal Equivalents (β)
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
a,b
a,b
b
b
CONTROL
HEAT
ETOH
COMPETITION
BACTERIA

Figure 2.2: The relationship between inbreeding depression and stress level for each population (Gala and Mayo), experimental set (Set I and II) and stressor. The level of stress is measured as the percent change (increase) in mortality of outbred individuals under stressful relative to benign conditions. The difference in the level of inbreeding depression under stressful (β_{stress}) versus benign conditions (β_{benign}) represents the change in the level of inbreeding depression under stress. The regression shown is constrained to pass through the origin, since by definition at zero stress $\beta_{stress} = \beta_{benign}$.

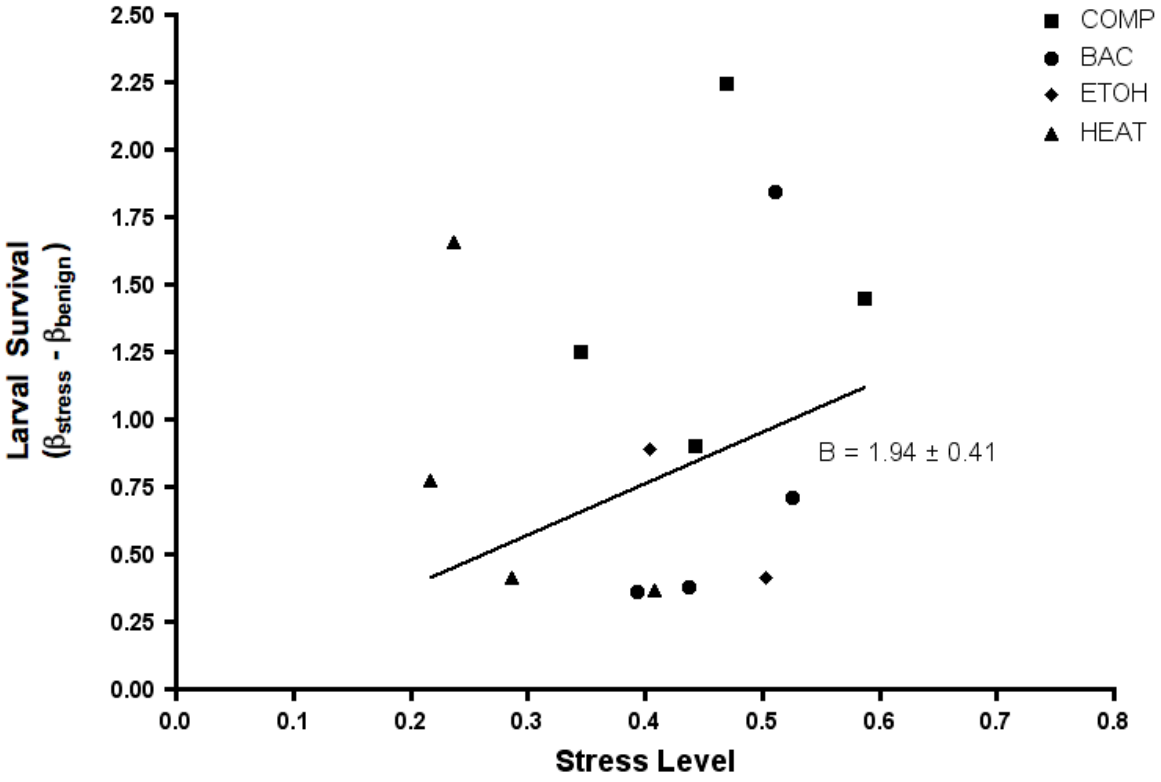
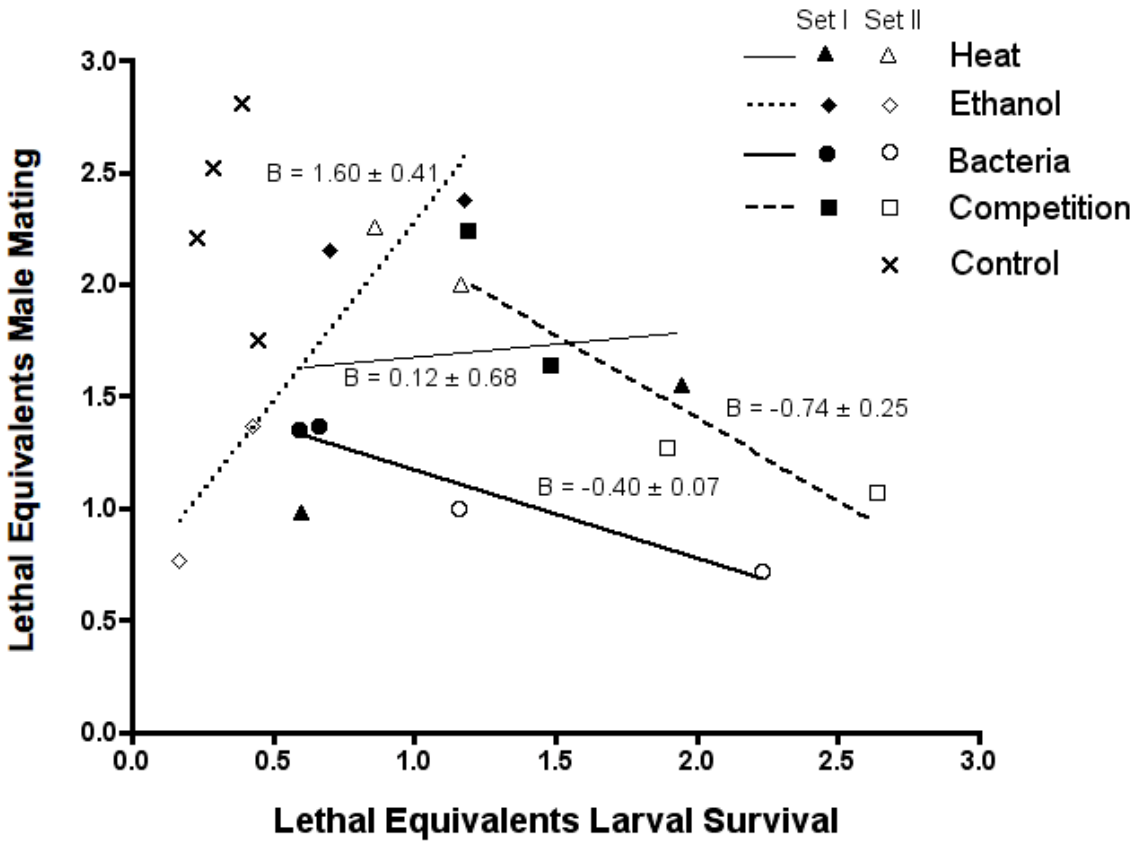

COMP
BAC
ETOH
HEAT
B = 1.94 ± 0.41
Larval Survival
(βstress - βbenign)
Stress Level
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Figure 2.3: Relationship between inbreeding depression (lethal equivalents) during the stressed larval period and post stress male mating success. Inbreeding depression was estimated using larval survival (LS not LCI) and the male competitive index (MCI). For each of the stress treatments four points are shown corresponding to the average lethal equivalents in each population (Gala and Mayo) within each replicate (Set I and II). The slope (B) is indicated for each stress.


Set I
Set II
Heat
Ethanol
Bacteria
Competition
Control
B = 1.60 ± 0.41
B = 0.12 ± 0.68
B = -0.74 ± 0.25
B = -0.40 ± 0.07
Lethal Equivalents Male Mating
Lethal Equivalents Larval Survival
0.0
0.5
1.0
1.5
2.0
2.5
3.0

Chapter 3

Sex-specific effects of inbreeding in wild-caught *Drosophila melanogaster* under benign and stressful conditions

Abstract

In animal populations, sib mating is often the primary source of inbreeding depression (ID). We used recently wild-caught *Drosophila melanogaster* to test if such ID is amplified by environmental stress and, in males, by sexual selection. We also investigated if increased ID due to stress (increased larval competition) persisted beyond the stressed stage, and if the effects of stress and sexual selection interacted. Sib mating resulted in substantial cumulative fitness losses (egg to adult reproduction) of 50% (benign) and 73% (stressed). Stress increased ID during the larval period (23% to 63%), but not during post-stress reproductive stages (36% to 31%), indicating larval stress may have purged some adult genetic load (although ID was uncorrelated across stages). Sexual selection exacerbated inbreeding depression, with inbred male offspring suffering a higher reproductive cost than females, independent of stress (57% vs. 14% benign, 49% vs. 11% stress).

Introduction

Inbreeding, or the mating of relatives, and the associated cost to fitness known as inbreeding depression, have long been a focus in evolutionary biology, ecology, and conservation biology (Charlesworth & Charlesworth, 1987; Ralls *et al.*, 1988; Keller & Waller, 2002; Kristensen & Sorensen, 2005). Inbreeding depression is a common phenomenon in nature and has been documented in a wide range of taxa (Crnokrak & Roff, 1999; Keller & Waller, 2002). As a result, inbreeding is recognized as a potent force influencing the persistence of natural populations (Keller & Waller, 2002; Spielman *et al.*, 2004; Frankham, 2008) as well as shaping the evolution of life history, morphology, physiology and behavior (Charpentier *et al.*, 2007). However, we have a poor understanding of the factors that contribute to the considerable variation in the severity of inbreeding depression that has been observed across taxa, populations, and even life history stages (Hedrick & Kalinowski, 2000; Keller & Waller, 2002; Pemberton, 2008). Currently, sexual selection and environmental stress have become the focus of much investigation due to the potential role they play in determining the magnitude of inbreeding depression. It has been hypothesized that sexual selection can reduce inbreeding depression by increasing selection against deleterious alleles (Radwan *et al.*, 2004; Jarzebowska & Radwan, 2009) while environmental stress often amplifies the negative effects of inbreeding on fitness (Armbruster & Reed, 2005). In general, both sexual selection and environmental stress are predicted to increase the effectiveness with which deleterious alleles

are purged from a population (Whitlock & Bourguet, 2000; Kristensen *et al.*, 2003; Swindell & Bouzat., 2006; Whitlock & Agrawal, 2009). However, it is unknown how these two forces interact to shape the genetic architecture and expression of inbreeding depression in natural populations.

Recent interest in the role of sexual selection in population persistence has centered around the potential for sexual selection to purge mutational load, thereby preventing mutational meltdown and reducing rates of extinction in small populations (Whitlock, 2000; Sharp & Agrawal, 2008; Jarzebowska & Radwan, 2009). While there is some evidence that sexual selection can effectively remove deleterious mutations from populations (Whitlock & Agrawal, 2009) much less is known about the effects of sexual selection on the severity of inbreeding depression. Sexual selection theory predicts that females who choose superior males increase the chances of survival of their offspring by selection of beneficial alleles and implies that males expressing deleterious alleles are less likely to find mates (Williams, 1966; Whitlock & Bourguet, 2000). Being inbred may therefore be more costly for males than females. Several non-*Drosophila* studies provide evidence that aspects of sexual selection such as male-male competition and female choice exacerbate inbreeding depression for components of male mating success and for lifetime reproductive success (Potts *et al.*, 1994; Pray *et al.*, 1994; Meagher *et al.*, 2000; Slate *et al.*, 2000; Höglund *et al.*, 2002). One study suggests that in *Drosophila,* the pattern may be similar (Miller & Hedrick, 1993). Inbreeding depression was significantly greater in males when sexual selection

was included as a component of male fitness (as measured by competitive male mating success). Miller and Hedrick (1993) reported substantially higher levels of inbreeding depression for competitive male mating ability (72.5%) than for female fecundity (1.5%, non-significant). This bias was not found when sexual selection was not incorporated as a component of male reproductive fitness (Robinson *et al.*, 2009).

Inbreeding depression is also widely recognized as being negatively affected by environmental stress. However, the magnitude of inbreeding depression reported under stressful conditions is highly variable (see Armbruster and Reed 2005). As a result several authors have argued that the current body of research demonstrates a lack of consistent or predictable effects of environmental stress on the expression of inbreeding depression (Keller & Waller, 2002; Armbruster & Reed, 2005; Waller *et al.*, 2008). Furthermore, most of this research has focused on overall survival or single fitness components, which has left another important question unanswered: what is the effect of early stress on inbreeding depression in later life history stages? It is unclear whether or not stress has a long lasting effect on the physiological functioning of an organism, exacerbating the effects of inbreeding on fitness even after the source of stress is removed. Two alternative hypotheses predict different changes in the level of inbreeding depression following exposure to stress during development. Environmental stress could amplify inbreeding depression in later life history stages due to greater vulnerability of inbred individuals to long lasting phenotypic

effects of stress. Alternatively, stress may purge genetic load during development, thus reducing levels of inbreeding depression in later life history stages. This can occur if stress increases selection against deleterious mutations that affect fitness at multiple life history stages (Haldane, 1957). This hypothesis predicts that stress will amplify inbreeding depression during the stage at which individuals are exposed (e.g. larval survival), but will have either no effect or reduce inbreeding depression for later performance (e.g. reproduction), as observed by Montalvo (1994) in the blue columbine (*Aquilegia caerulea*). These alternatives are not mutually exclusive, but it important to understand their relative importance.

In *D. melanogaster,* given that females exhibit strong sperm precedence (see review in Manier et al. 2010) and lay multiple eggs on a single fruit (Nunney, 1990), full sib mating is expected to occur in the wild among offspring from a single fruit. However, despite the extensive use of *D. melanogaster* as a model for the effects of inbreeding on fitness, an accurate measure of the cost of sib mating in wild populations is lacking. The vast majority of studies have used populations maintained under laboratory conditions for well over 20 generations (Tantawy, 1957; Sharp, 1984; Mackay, 1985; Miller *et al.*, 1993; Miller & Hedrick, 1993; Garcia *et al.*, 1994; Hughes, 1995; 1997; Fowler & Whitlock, 2002; Hughes *et al.*, 2002). The few exceptions are those studies that have measured the fitness effects of individuals made homozygous for various chromosomes extracted from males taken directly from wild populations. Laboratory adapted

populations may have a different genetic architecture relative to natural populations, for example due to bottleneck events occurring during the establishment or maintenance of a laboratory population. This may result in differences in levels of inbreeding depression. Concerns have been raised regarding potential underestimation of the levels of inbreeding depression found in nature (Sheilds, 1993; Hedrick & Kalinowski, 2000; Joron & Brakefield, 2003) when lab populations are used. In order to investigate the effect of inbreeding in flies with a natural genetic architecture the cost of sib mating needs to be measured from recently wild caught populations.

The aim of this study was to evaluate how inbreeding depression is influenced by both sexual selection and environmental stress using two wild caught populations of *D.melanogaster.* Specifically this study addresses three main questions: 1) How large is the fitness cost of sib mating when developmental and reproductive costs are included? 2) Are reproductive costs in the inbred offspring greater in males than females? 3) Given a stressful environment during larval development, does inbreeding depression increase for larval survival and/or adult reproduction? The cost of one generation of full sib mating was measured in two populations of *D.melanogaster* collected from Northern California after only 1 generation of controlled outcrossing in the laboratory (to avoid any unintentional purging of deleterious recessive alleles). We measured egg hatchability, larval to adult survival, female fecundity, and male mating success of inbred offspring, and evaluated the role of stress on

inbreeding depression by comparing the effect of rearing larvae under conditions of low and high food competition. In addition, we evaluated the effect of the age of females on inbreeding depression for female fecundity by measuring the number of offspring produced separately during the early (days 1-8) and late (days 8-16) stages in the female's life. Previous work in *Drosophila* has shown that inbreeding depression increases with age (Hughes, 1995; Hughes *et al.*, 2002).

Methods

Base population and inbreeding design

D. melanogaster populations were collected from two locations in Northern California, the Galante Winery in Carmel Valley (Gala) and the Mayo Family Winery in Sonoma Valley (Mayo). In order to prevent modifying the genetic architecture through selection or inbreeding, 400 pairs of wild caught flies from each location were placed in single vials and reared in the laboratory for one generation. Their progeny were outcrossed by taking a single male and female from each of the 400 pairs (per population) and mating them in a circular design, whereby each male is mated to the female from the next vial. The resulting outrossed progeny (P generation) were then used as parents to establish families in the breeding design explained below. The initial crosses ensured that each of the original pairs contributed an equal number of progeny, that all P generation

flies from a single vial were full-sibs, and that no inbreeding occurred since capture.

The P generation was created by collecting fifty-six virgin full sibs, twenty eight of each sex, from each of 16 Gala vials and 12 Mayo vials (each vial constitutes one outbred family). For each family, these P generation flies were divided into two equal groups (14 of each sex) in order to create both inbred (IB) and outbred (OB) crosses. For inbred crosses, 14 virgin females were sib-mated to 14 of their virgin brothers in a single vial and for outbred crosses 14 virgin females were mated to 14 virgin males from another family that was chosen at random without replacement. For example, females (all sisters) from Gala family A were crossed to their brothers from family A to create the inbred cross. In addition, this same family was involved in two outbred crosses: A♀ x C♂ (the maternal cross of family A) and D♀ x A♂ (the paternal cross). The results from A♀ x A♂ and A♀ x C♂ made up a maternal family lineage and the results from A♀ x A♂ and D♀ x A♂ made up a paternal family lineage. The 16 Gala families and 12 Mayo families are a subset of the original 400 pairs that were round-robin crossed to create the P generation. As a result, the round-robin design was not complete for the families tested and therefore the number of maternal (28 total) and paternal (20 total) lineages used in subsequent analyses were unequal. All experiments were carried out at 25°C.

Inbreeding depression (δ) was calculated for each family as the loss of fitness exhibited by the progeny of a sib mating, i.e. $1 – (w_{inbred} / w_{outbred})$, where $(w_{inbred} / w_{outbred})$ is the fitness of the progeny from the sib mated cross relative to the outbred cross from each family. With the design described above, inbreeding depression can be calculated separately for the maternal and paternal lineages. For example, the value of $w_{outbred}$ used to calculate inbreeding depression for family A could be either from the maternal (A♀ x C♂) or the paternal (D♀ x A♂) outbred cross. Note that the maternal data and the paternal data sets are each internally independent, but that they are not independent of each other.

1) Egg hatchability:

For each family cross (IB and OB), groups of 14 males and 14 females were set up in an empty glass bottle capped with a petri dish containing standard food medium and allowed to lay eggs for a period of 8 hours. The groups were transferred to new laying dishes every 8 hours until at least 350 eggs had been laid per family cross. The number of eggs laid were counted and the number of unhatched eggs were counted 24 hours later. Inbreeding depression was calculated per family as δ = (1 – (% hatchedinbred / % hatchedoutbred)) for both maternal and paternal lineages.

2) Larval-adult survival:

Larval to adult survival was measured on two concentrations of food (1x and 1/3x) in the presence of larvae from a standard competitor laboratory stock

(*spa)*. The *spa* competitor flies have a recessive sparkling eye phenotype that can be distinguished from the wild type test flies. The 1x concentration of food is the standard food medium consisting of molasses, cornmeal, yeast, water and the antifungal agent Tegosept. The 1/3x concentration of food was created by adding 2/3 agar (18g/L) to 1/3 of the standard 1x food medium and was chosen based on preliminary experiments demonstrating an average 45% reduction in survival relative to the standard 1x concentration of food.

First instar larvae were collected in groups of 50 larvae per family for up to 8 hours from the above described laying dishes. This ensured that all larvae were at ± 4 hours apart in development. Larvae were transferred using a paintbrush (ID #000) to vials containing 10ml of food medium (1x or 1/3x) in the ratio of 50 test line to 150 of the standard competitor *spa*. Three replicate vials of each concentration of food (1x and 1/3x) were set up for each family cross (IB and OB for the 16 Gala and 12 Mayo families) and placed at 25°C.

Following the set up of first instar larva in vials (day 1), once the first eclosed progeny were observed (days 9-10) all emerging adults were counted and removed every 3-4 days until approximately day 20-21, by which time the number of first generation progeny emerging per vial had diminished to zero (or nearly so) over the final 3-4 day counting interval (day ~19-21) and a large number of dark pupae representing the next generation were observed. There was always a clear distinction between the first and second generation.

Larval-adult survival was measured in two ways: as the proportion of test

larvae surviving to eclosion (LS) and as the larval competitive index (LCI), which is the proportion that eclosed per vial of the test line relative to the proportion of *spa* competitors (Knight & Roberston, 1957). Inbreeding depression was calculated as (1 – (LSinbred / LSoutbred)) and (1 – (LCIinbred / LCIoutbred)) for both maternal and paternal lineages.

3) Fecundity of female offspring:

Adult F_1 females that emerged from the larval-adult survival assay were collected and used to measure female fecundity. Virgin females were collected from each replicate vial of food concentration (1x and 1/3x) and breeding treatment (OB and IB) and placed at 25°C until they were 4-7 days old. Three females were randomly selected from each vial and each was placed individually with two unrelated males from a standard outbred laboratory strain (MEL) for 48 hours at 25°C. Single females were transferred without males to a new vial with standard food medium and allowed to lay eggs for 8 days (vial 1), after which each female was transferred again to a new vial and allowed to lay for an additional 8 days (vial 2).

Female fecundity was measured in three ways for each individual female: 1) early fecundity from vial 1 (days 1-8) where the females were 11-14 post eclosion at the end of the laying period; 2) late fecundity from vial 2 (days 9-16) where females where 19-22 days post eclosion at the end of the laying period; and 3) total progeny production over 16 days (sum of progeny from both vials).

Note that these females (both inbred and outbred) were outcrossed so that their offspring would not exhibit inbreeding depression. As with the larval-adult survival assay, care was taken to avoid including a second generation in the progeny counts. Inbreeding depression was calculated as (1 – (avg # progenyinbred females / avg # progenyoutbred females)) for both maternal and paternal lineages. Females that were collected for testing but subsequently died or failed to produce offspring were included as having zero fecundity.

4) Mating success of male offspring:

Adult F_1 males that emerged from the larval-adult survival assay were used to measure male mating success. Virgin males were collected from each replicate vial and placed at 25°C until they were approximately 14-18 days old. Five males (full sibs) were randomly selected from each vial and placed with 15 unrelated virgin competitor *spa* males and 10 unrelated virgin *spa* females in new vials containing 10ml food. In order to control for the quality of competitor flies, *spa* males and females used in these mating trials were reared at low densities in 30 bottle populations of ~200 adults each at 25°C and transferred every 3-4 days to avoid overcrowding. The mating trials lasted for two hours at 25°C, after which females were removed using light anesthesia and transferred individually to new vials. Preliminary experiments determined that this time period and ratio of test males to competitor males minimizes multiple mating and maximizes the overall number of successful matings, which is in agreement with previous studies

(Sharp, 1984; Miller & Hedrick, 1993). After ~2 weeks the progeny of each *spa* female were scored for eye color to determine her mate. The *spa* phenotype is recessive and therefore if a *spa* female mates to a test male 100% of the progeny will be wild type for eye color. The short mating period (2hrs) was designed to avoid females mating to multiple males and the ratio of test males to *spa* males (5:15) provided a level of male competition that minimized the number of trials where one type of male did not mate to any of the females. A random subset of 5 families per population was selected for measurement.

Male mating success was measured both as a proportion of test males mating (MS), and relative to the standard competitor *spa* as the male competitive index (MCI). MCI was defined as the proportion of females inseminated by test males divided by the proportion of females inseminated by the standard competitor *spa* males. Inbreeding depression was calculated as (1 – (MSinbred male */* MSoutbred male)) and (1 – (MCIinbred male */* MCIoutbred male)) for both maternal and paternal lineages.

5) Cumulative Inbreeding Depression

Cumulative inbreeding depression across all life history traits was calculated separately for both males and females and separately using either maternal and paternal lineages as the outbred reference (see above). By multiplying % egg hatchability (EH), the larval competitive index (LCI), and either the 16-day total female fecundity (TFF) or the male competitive index (MCI),

cumulative average fitness was calculated for each inbred (W_{inbred}) and outbred ($W_{outbred}$) line in both sexes. This assumes EH and LCI are equal in the two sexes, as found by (Frankham & Wilcken, 2006).

Cumulative inbreeding depression is expressed as:

$1 - (EH \times LCI \times TFF_{inbred} / EH \times LCI \times TFF_{outbred})$ for females

$1 - (EH \times LCI \times MCI_{inbred} / EH \times LCI \times MCI_{outbred})$ for males

6) Lethal equivalents

For each fitness trait as well as cumulative male and female fitness, we calculated β, the number of lethal equivalents per haploid genome as:

$$\beta = -[\ln(w_{inbred} / w_{outbred})]/F$$

(Morton *et al.*, 1956) where F is the level of inbreeding (F = 0.25). The number of lethal equivalents is defined by the rate at which the logarithm of fitness declines with inbreeding. This method is commonly used to compare the effects of inbreeding on fitness across studies, species/taxa, and environments (Hedrick & Kalinowski, 2000; Armbruster & Reed, 2005).

Statistical Analysis

Two separate analyses were run, one where family was assigned according to maternal lineage and one according to paternal lineage for all fitness measures (see explanation above). Larval survival, female fecundity and male mating success were analyzed using an ANOVA (SAS Version 9.1 for Windows ®) with the following variables: INBREED (inbred vs outbred), COMP (high vs low larval competition), POP (Gala, Mayo), FAM (P generation family nested within population, using either the female or male lineage in separate analyses to define the outbred cross), plus all two and three way interactions involving INBREED and COMP. INBREED and COMP are fixed effects while POP and FAM are random effects. Egg hatchability was analyzed using the above model but without the variable COMP. Both competitive indices (LCI and MCI) were arcsine square root transformed so that values were more normally distributed. For female fecundity a two separate analyses were run 1) and ANOVA on total fecundity and 2) a repeated measures ANOVA using procedure MIXED on early and late fecundity. Unless otherwise stated, results reported are those for total female fecundity. Finally, pair-wise correlations were calculated between larval survival (LCI) and each adult fitness measure (FF, MCI) across families in both inbred and outbred individuals separately. In addition, the competitive index and raw percentage values were correlated for larval survival (LCI and % survival) and male mating success (MCI and % matings). Pair-wise

correlations were also calculated for inbreeding depression in larval survival, female fecundity and male mating success.

Cumulative male and female relative fitness, calculated as ([EH x LCI X $MCI)_{males}$ or $(EH \times LCI \times FF)_{females}$, was analyzed in the families that had data across all four life history traits using the following ANOVA model: COMP, FAM, SEX, POP and all interactions where COMP and SEX were fixed effects and POP and FAM are random effects.

Interactions with a p value greater than 0.25 (Kirk, 1982) were removed from the above models and the analysis was rerun. When a significant interaction occurred in the original model, the analysis was split (for example by POP or COMP) and a Bonferoni correction was used to adjust for multiple testing.

Results

The data were analyzed for each life history stage separately and cumulatively across all stages. The analysis is presented first using the maternal lineage as the outcross reference (e.g. comparing A♀ x A♂ offspring to A♀ x C♂ offspring, see Methods). The robustness of these results is then examined by presenting the analysis based on the paternal lineage (e.g. comparing A♀ x A♂ offspring to C♀ x A♂ offspring).

Maternal Family Analysis

Inbreeding in egg hatchability caused a small but significant overall reduction in the Mayo population ($F_{1,11} = 16.67$ $p < 0.01$, Table 3.3.1a) and there was significant variation in the magnitude of the inbreeding depression among families in both populations (Gala $F_{15,152} = 6.96$ $p < 0.001$, Mayo $F_{11,113} = 3.82$ $p < 0.01$). The overall inbreeding depression (< 2%), corresponded to less than 0.1 lethal equivalents in both populations (Table 3.3.1b).

Inbreeding had a much larger effect on larval survival ($F_{1,25} = 136.12$ $p < 0.001$; Table 3.3.2) of 19% under benign conditions. Taking into account the response of competitors, this estimate increased to 23%, as measured by the larval competitive index (LCI). Under stressful conditions of limited food (high larval competition), inbreeding depression was higher, at 34% (% survival) and 63% (LCI). Based on LCI this corresponds to a shift from roughly 1 to 4 lethal equivalents (Table 3.3). There were no significant differences between the populations in any of these measures and, although there was significant variation across families for larval success ($F_{24,289} = 1.87$ $p < 0.05$; Table 3.3.2), there were no differences among families in inbreeding depression.

Male mating success and female fecundity were both reduced due to inbreeding ($F_{1,18} = 14.03$ $p < 0.01$ and $F_{1,26} = 21.54$ $p < 0.001$; Table 3.3.2) regardless of population. Unlike larval survival, inbreeding depression in these traits was not amplified by rearing under high larval competitive stress (Table 3.3.1) even though the absolute levels of fecundity and male mating success

were substantially reduced (Table 3.3). Inbred females produced on average 20 (13%) fewer total offspring than outbred females, i.e. 0.56 lethal equivalents (Table 3.3). However, when fecundity was divided into early (age: 6-14 days) and late (age: 15-22 days) stages inbreeding depression differed between the stages ($F_{1,1319} = 69.36$ $p < 0.001$;Table 3.4). While early female fecundity was not different between inbred and outbred females, significant inbreeding depression was found for late female fecundity (0.98 lethal equivalents, Tables 3 and 4). Inbreeding depression for female fecundity did not differ when females with zero fecundity that died or failed to produce offspring (12 inbred, 6 outbred) were included versus when they were removed. Inbred males had 29% lower mating success compared to outbred males (corresponding to 1.37 lethal equivalents), a difference that rose to 54% (3.11 lethal equivalents) when their success relative to the *spa* males was taken into account (Table 3.3). Males were 14-18 days old when tested, so male inbreeding depression was greater than that found in older and younger females. Levels of inbreeding depression varied significantly across families for total female fecundity ($F_{26,801} = 2.73$ $p < 0.001$), but not for male mating success (Table 3.3.2).

The larval and male fitness components were estimated directly as percent success or relative to standard competitors. These measures were highly correlated for both inbred and outbred offspring combined under conditions of low larval competition (larval survival: $r = 0.95$, d.f. = 52, $p < 0.001$, male mating: $r = 0.77$, d.f.= 25, $p < 0.001$). These correlations were lower given high larval

competition, especially for male mating (larval survival: r = 0.79, d.f.= 49, p< 0.001, male mating: r = 0.42, d.f.= 24, p< 0.05).

The three fitness components (larval survival, female fecundity and male mating) were not correlated across the outbred families: the highest correlation was between larval survival (LCI) and male mating success (MCI) under high competition (r = 0.76, d.f. = 8, p< 0.05 uncorrected for multiple testing). Furthermore, inbreeding depression was not significantly correlated across the families/lineages for larval survival, female fecundity and male mating success within or between larval competitive treatments (all p> 0.1 uncorrected for multiple testing). In particular, there was no change in the correlation between larval inbreeding depression and the inbreeding depression in male or female reproduction under benign (r = -0.09, 0.01; df = 8, 24) vs. stressed (r = -0.06, 0.13, df = 8, 13) conditions, as might be expected if there was larval-stage purging of genetic load affecting adult traits and if this purging increased under stress.

Comparison of male and female reproductive fitness of the outcrossed families showed non-significant negative correlations (r = -0.58, -0.21 under benign and stress conditions). Inbreeding depression showed non-significant positive correlations (r = 0.51, 0.30; df = 7, 8).

Combining the developmental effects of hatchability and larval survival with adult reproduction (averaged across the sexes) results in a cumulative fitness loss for a sib-mated pair of 50% (2.77 lethal equivalents) under benign

conditions, and 73% (5.24 lethal equivalents) if their offspring experienced high larval competition (Table 3.5). The high inbreeding depression for male reproduction (mating success) relative to female reproduction (fecundity) resulted in cumulative inbreeding depression being significantly higher for male offspring than female offspring under both standard conditions (low competition) and limited food (high competition) (Figure 3.1, Table 3.5). This sex difference was not significantly altered by larval environment (Table 3.5).

Paternal Family Analysis

The paternal analysis differs from the maternal approach in having the potential to create an apparent family by inbreeding interaction (FAM x INBREED), since inbred and outbred families differ in maternal environment and the sons differ in the X-chromosome that they carry. However, the effect of sib mating on fitness was qualitatively the same as the maternal analysis in almost all respects (see Tables 1-4). Differences that generated shifts in significance were observed in egg hatchability, high larval competition, male mating success, and cumulative fitness. However, all inbreeding-related factors that were highly significant under the maternal analysis ($p < 0.01$) remained so under the paternal analysis. One of these (affecting male mating success) was due to an increase in the family by inbreeding interaction ($F_{10,97} = 2.09$ $p < 0.05$; Table 3.3.2), but conversely the influence of family on the cumulative inbreeding depression became non-significant (Table 3.5).

Discussion

This study demonstrates a number of important features of the relationship between inbreeding and fitness under conditions of sexual selection and environmental stress. Under standard lab conditions, sib mating resulted in a 50% cost to overall fitness in recently wild-caught *D. melanogaster* populations, corresponding to $\beta = 2.77$ lethal equivalents (Table 3.5). In addition, a striking difference was found in the level of inbreeding depression expressed in males and females (Figure 3.1). Inbred males suffered an almost 2-fold higher cumulative loss in fitness than females, a result consistent with the study of Miller and Hedrick (1993). This difference between the sexes remained the same under high larval competition (Table 3.5), while the overall fitness cost to sib mating increased. When these cumulative effects are broken down, we found that egg hatchability was only slightly affected by inbreeding (< 2%) while even under benign conditions, relative larval survival dropped by about 20%. We also found that larval competitive stress amplified this larval inbreeding depression but it did not increase inbreeding depression in the later life history stages of adult reproduction. This result argues against the hypothesis that stress induces long-lasting negative phenotypic effects in inbred individuals, but may be consistent with the possibility that stress, through purging some of the genetic load, can lower inbreeding depression at a later stage.

All of these patterns were consistent across the two replicate populations. The only significant (but trivial) difference between them was a 2% level of inbreeding depression for egg hatchability in the Mayo population vs. 1% in Gala.

Magnitude of Inbreeding Depression in Recently Wild-caught D.melanogaster

Table 3.6 summarizes literature on the effects of inbreeding on individual fitness components in *D. melanogaster* measured under standard benign laboratory conditions using fitness measures directly comparable to those measured in this study (see Simmons and Crow (1977) and Charlesworth and Charlesworth (1987) for reviews of estimates based on chromosomal homozygote populations). Only two studies (Miller & Hedrick, 1993; Robinson *et al.*, 2009) measured inbreeding depression across the full spectrum of life history stages in both sexes, and Robinson *et al.* (2009) excluded sexual selection in males. Our finding that mating between siblings caused a 50% reduction in overall cumulative fitness in recently caught populations of *D. melanogaster* under standard laboratory conditions (Figure 3.1, Table 3.5) is consistent with results of Miller and Hedrick (1993). We found that rearing conditions that included larval competitive stress resulted in a larger 72% reduction in overall fitness.

The contribution of egg hatchability to cumulative inbreeding depression was statistically significant but very small (2%), similar to the 6% found by Biémont (1978). The very low inbreeding depression in egg hatchability suggests

that a limited fraction of the offspring's genes are expressed at this stage and/or that mutations in the genes involved in early development are generally not fully recessive, limiting the mutation-selection build-up of deleterious alleles. In contrast, reductions in fitness under low and high larval competition respectively in larval to adult survival (22%, 63%), the fecundity of female offspring (15%, 11%) and the mating success of male offspring (51%, 48%) were all substantial. Overall, our estimates of lethal equivalents for larval-adult survival (0.95) and female fecundity (0,6-14 days; 0.98, 15-22 days) are similar to those previously reported (Table 3.6). The finding that inbreeding depression for female fecundity increased with female age (Table 3.4) is in agreement with several studies demonstrating significant age effects in both females and in males (Hughes, 1995; Hughes *et al.*, 2002).

Inbreeding depression is expected to vary among families, since they represent a random sampling of deleterious alleles in the population (Hedrick & Kalinowski, 2000; Haag *et al.*, 2003). Interactions between inbreeding and families/lineages have been found in *Drosophila* (Bijlsma *et al.*, 1999; Dahlgaard & Hoffmann, 2000; Reed *et al.*, 2003), *Daphnia* (Haag *et al.*, 2003), *Peromyscus polionotus* (Lacy *et al.*, 1996) *Tribolium castaneum* (Pray & Goodnight, 1995), and plants (Dudash *et al.*, 1997; Byers & Waller, 1999). In this study, highly significant interactions between inbreeding and family were observed for egg hatchability and female fecundity (Tables 1 and 2). However, this among family variance is expected to decrease with an increase in the number of loci

contributing to the trait (and hence potentially a source of deleterious alleles), a pattern found in this study for larval competition, both under low and high competition conditions, and male mating success (Table 3.3.2). This lack of a family effect for larval performance and male mating success may indicate the involvement of a large number of mildly deleterious alleles, which minimizes the sampling variance among the families.

Comparing among the traits, our results suggest that alleles having deleterious effects on the fitness of inbred larvae do not affect later life history stages and/or that some deleterious alleles are purged at the larval stage so that they are not expressed at the later stages of female fecundity and male mating success. Under both low and high larval competition, the correlations between larval and adult traits were within the range 0-0.13. In addition, the non-significant positive correlation between male and female adult inbreeding depression under both benign and stressed larval conditions is consistent with the hypothesis that deleterious alleles show no consistent pattern in determining male mating success verses female fecundity.

Inbreeding Depression is Greater in Males

Inbred male offspring showed a substantially greater loss of fitness than females, regardless of the female age (late female fecundity, β= 0.98; male mating success, β= 3.01, Table 3.6). Assuming no sex differences in egg to adult survival (Frankham & Wilcken, 2006), the cumulative relative fitness of inbred

females was almost two fold higher than inbred males (Table 3.5, Figure 3.1), results similar to those of Miller and Hedrick (1993) (Table 3.6). In contrast, Robinson et. al. (2009) did not incorporate sexual selection into the measure of adult male reproductive fitness, and found no differences between the sexes in levels of inbreeding depression (both ~13%).

Marked sex differences in inbreeding depression have also been found in other animals. In house mice, Potts et. al. (1994) found significant inbreeding depression for the acquisition of territories by males while there was no detectable inbreeding depression for female fitness. In addition, the cost to sib mating in wild caught mice has been shown to be almost 4 times greater for inbred males than inbred females under semi-natural conditions (Meagher *et al.*, 2000). Pray et al. (1994) also report that male red flour beetles (*Tribolium castaneum*) suffer greater costs of being inbred than females for proportion of offspring produced in a competitive social environment. Such results suggest that sexual selection via male-male competition and/or female choice may be responsible for amplifying the effects of inbreeding on male fitness. However, sexual dimorphism in inbreeding depression may result from competition for resources (food, water, territory) in general and therefore will not always be male biased. For example, wild female song sparrows exhibit greater inbreeding depression for lifetime reproductive success than males (Keller, 1998).

A finding that selection is greater against inbred males than inbred females can have important implications for the role of sexual selection in

reducing genetic load in populations via female choice. Sexual selection is predicted to reduce the frequency of deleterious alleles in a population if males that carry a greater number of deleterious alleles are less likely to mate due to female choosiness (Whitlock & Bourguet, 2000), potentially reducing the risk of extinction in small populations (Whitlock, 2000). Recent empirical work in the bulb mite demonstrated that sexual selection can reduce both extinction rate and levels of inbreeding depression for small bottlenecked populations (Jarzebowska & Radwan, 2009) and a few other studies have demonstrated the effective removal of deleterious alleles from populations via sexual selection (Radwan, 2004; Radwan *et al.*, 2004; Sharp & Agrawal, 2008; Hollis *et al.*, 2009). However, our results illustrate a potential problem for the purging hypothesis, since it is assumed that deleterious alleles driven to a lower frequency by sexual selection results in an overall fitness benefit in females or in juveniles. We found no significant correlation between inbreeding depression in male reproduction and other traits. More empirical work is needed to determine if sexual selection can in general alleviate mutational load in populations.

Inbreeding Depression and Environmental Stress

Although we found that environmental stress (larval competition) increased inbreeding depression, we found no correlation between the benign and competitive environments in the inbreeding depression for larval survival (LCI r = -0.24) or in cumulative inbreeding depression (r = -0.09 female, r = -0.05

male). Several studies examining how purging of genetic load in different environments affects extinction rates suggest that environmental stress may increase the effectiveness of purging (Bijlsma *et al.*, 2000; Swindell & Bouzat., 2006). However, the implications for population persistence are unclear if purging is environment specific and purging provides no fitness benefit in novel environments (Bijlsma *et al.*, 1999; Leberg & Firmin, 2008).

A related phenomenon occurs when an environmental stress affects only one life history stage of an organism. Does this stress amplify inbreeding depression across the entire life cycle of an organism or does it reduce inbreeding depression in later life history stages due to genetic purging due to greater selection against individuals expressing deleterious recessive alleles (Armbruster & Reed, 2005; Waller *et al.*, 2008)? We tested these hypotheses by measuring inbreeding depression during exposure to stress and after the stressor has been removed. We found that competitive stress only amplified inbreeding depression during the stage at which it was applied. Inbreeding depression affecting larval survival was substantially increased under conditions of competitive resource stress (Table 3.3, Figure 3.1); however, female fecundity and male mating success, measured after the stress was applied, did not show increased inbreeding depression (Tables 2 and 3, Figure 3.1). It is possible that the purging of individuals with the highest genetic load at the stressful stage (larval to adult development) could explain why inbreeding depression in the adult fitness stages was not increased (Table 3.3). However, the evidence for

purging is weak since the among-family correlation linking inbreeding depression at the larval and reproductive stages is very close to zero under both benign and stressed conditions. This does not exclude the possibility of a purging effect, but for purging at an early stage to increase later fitness requires that some of the same deleterious alleles affected both stages, a scenario that would typically generate a positive correlation between inbreeding depression at the two stages under benign conditions.

Work in the blue columbine (*Aquilegia caerulea*) suggested that exposure to harsher conditions early in life (field vs. greenhouse germination) may lower inbreeding depression at later stages (Montalvo, 1994). This is presumably due to a reduction in selection against deleterious alleles under benign greenhouse conditions during the seedling period. Several other studies in plants show similar patterns of greater inbreeding depression for early life history (seed survival) than adult fitness (plant size) when seeds experienced stressful field conditions (Schoen, 1983; Kohn, 1988). Note that this role of purging cannot be detected in studies comparing lines or families that are genetically identical (e.g. when specific chromosomes are made homozygous).

Implications for Inbreeding Avoidance in D. melanogaster

In general, individuals that employ mechanisms to avoid mating with relatives have a selective advantage over those that do not, driving the evolution of mechanisms to avoid inbreeding (Pusey & Wolf, 1996; Panhuis & Nunney,

2007). This is because the genetic load of recessive deleterious alleles that causes inbreeding depression also creates conditions that favor genotypes that avoid inbreeding. Little is known about levels of full sib mating and the associated fitness costs in wild populations of *Drosophila*, the two factors that determine the strength of selective forces driving the evolution of avoidance mechanisms. In two cactophilic species of *Drosophila* and in *D. melanogaster* it has been observed that females appear to reduce sperm use from related males, which may be beneficial by reducing inbreeding depression in their offspring (Markow, 1997; Panhuis & Nunney, 2007). Several other studies provide circumstantial evidence supporting the existence of postmating, prefertilization inbreeding avoidance (PPIA) in *D. melanogaster.* For example, sperm competitive ability has been shown to decrease with the degree of relatedness between males and females (Clark *et al.*, 1995; Clark & Begun, 1998; Clark *et al.*, 1999; Mack *et al.*, 2002). Our work clearly demonstrates massive fitness costs of mating with a sibling (Table 3.5, Figure 3.1) in recently caught *D. melanogaster* especially if there is larval competition. Larval competition is found in nature (Nunney, 1990) and such large fitness costs provide a strong selective environment in which PPIA could evolve in this species. The level of sib mating is still unknown in wild populations of *D.melanogaster*, however, it appears that flies generally mate before dispersing from their natal site (unpublished data) so the frequency of such matings could be significant.

Finally, the way in which inbreeding depression is measured, using either a competitive index (LCI or MCI) versus using uncorrected percent survival or mating success, is important in an ecological context. Estimating inbreeding depression using a competitive index is representative of situations in nature where multiple females lay eggs on a single fruit. Under these conditions the inbred offspring of a female that has mated to a sibling are potentially competing against outbred offspring from other females. Alternatively, if only a single female lays eggs on a fruit then raw percent values would be an appropriate measure of fitness since inbred offspring are only competing with other inbred offspring. Inbreeding depression would be expected to be less under these conditions compared to conditions where multiple females lay eggs on a single fruit.

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Tables and Figures

Table 3.1: Analysis of inbreeding depression in egg hatchability. Outcrossing was evaluated either via the maternal lineage or via the paternal lineage. A) Analysis of variance of the fitness loss due to sib mating versus outcrossing (INBREED). The model also included population Gala vs. Mayo (POP) and family (FAM). Analysis was split by population due to a significant interaction between INBREED and POP ($F_{1,168}$= 3.12 $p < 0.001$). B) Average (± se) egg hatchability, inbreeding depression (δ) and number of haploid lethal equivalents (β) in the two populations (Gala and Mayo). *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$.

A) ANOVA EGG HATCHABILITY

Maternal Lineage		**GALA**		**Paternal Lineage**			
Source	*df*	*MS*	*F*†	*Source*	*df*	*MS*	*F*†
INBREED	1	0.0682	4.00	INBREED	1	0.0223	1.50
FAM	15	0.0184	8.02***	POP	1	0.0405	3.00
INBREED X FAM	15	0.0160	6.96***	FAM(POP)	17	0.0137	6.12***
Error	152	0.0023		INBREED X FAM(POP)	17	0.0171	7.63***
		MAYO		INBREED X POP	1	0.0004	0.17
Source	*df*	*MS*	*F*†	Error	183	0.0022	
INBREED	1	0.1650	16.67**				
FAM	11	0.0068	2.62**				
INBREED X FAM	11	0.0099	3.82**				
Error	113	0.0026					

† **F Ratios.** Unless specified below F ratios calculated as MS_{effect}/MS_{error}

Effect	*F Ratio*
$INBREED_{Maternal}$	$MS_{INBREED}/(MS_{FAMxINBREED})$
$INBREED_{Paternal}$	$MS_{INBREED}/(MS_{POPxINBREED} + MS_{FAMxINBREED} - MS_{error})$
$POP_{Paternal}$	MS_{POP}/MS_{FAM}

B) EGG HATCHABILITY

Population	**OUTBRED**	**INBRED**	**δ**	**β**
GALA				
Maternal	97.70 ± 0.53	96.62 ± 0.42	0.009 ± 0.04***	0.04
	N= 98	N= 88		
Paternal	98.40 ± 0.18	95.64 ± 1.06	0.020 ± 0.01***	0.10
	N= 57	N=47		
MAYO				
Maternal	98.94 ± 0.09	97.10 ± 0.27	0.019 ± 0.01***	0.08
	N= 73	N= 64		
Paternal	98.91 ± 0.15	97.20 ± 0.40	0.020 ± 0.01***	0.07
	N=62	N=55		

Table 3.2: Analysis of variance of the fitness loss due to sib mating versus outcrossing (INBREED) for larval survival, total fecundity of surviving females, and mating success of surviving males. Outcrossing was evaluated either via the female lineage or via the male lineage. The model also included population Gala vs. Mayo (POP), high vs. low competition (COMP), and family (FAM). A) Larval survival. The analysis was split by level of competition due to a significant interaction between INBREED and COMP in the initial analysis ($F_{1,523}$= 129.83 $p < 0.001$). B) Fitness measures of adult offspring. *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$.

A. LARVAL SURVIVAL UNDER DIFFERENT LEVELS OF LARVAL COMPETITION

Low Larval Competition		**Maternal Lineage**			**Paternal Lineage**	
Source	*df*	*MS*	*F*†	*df*	*MS*	*F*†
INBREED	1	0.2586	136.12***	1	0.0855	35.63***
POP	1	0.0018	0.93	1	0.0020	0.85
FAM(POP)	25	0.0023	1.24	21	0.0024	0.82
INBREED X FAM(POP)	25	0.0019	1.04	22	0.0024	0.81
Error	290	0.8102		77	0.003	
High Larval Competition						
Source	*df*	*MS*	*F*†	*df*	*MS*	*F*†
INBREED	1	5.2523	187.60***	1	1.8005	118.50***
POP	1	0.0002	0.01	1	0.0031	0.11
FAM(POP)	24	0.0373	1.87*	19	0.0270	1.63
INBREED X FAM(POP)	23	0.0280	1.45	20	0.0152	0.91
Error	289	0.0200		76	0.0166	

B. ADULT FITNESS MEASURES

FEMALE FECUNDITY		**Maternal Lineage**			**Paternal Lineage**	
Source	*df*	*MS*	*F*†	*df*	*MS*	*F*†
INBREED	1	51316.3	21.54***	1	49395.7	22.36***
POP	1	311.4	0.16	1	14.1	0.001
FAM(POP)	27	1971.5	2.26**	16	2124.1	2.70**
COMP	1	62708.4	71.92***	1	26596.5	33.80***
INBREED X FAM(POP)	26	2382.4	2.73***	17	2209.3	2.81**
INBREED X COMP	1	2007.8	2.3	1	3.2	0.01
Error	801	871.9		464	786.8	
MALE MATING SUCCESS						
Source	*df*	*MS*	*F*†	*df*	*MS*	*F*†
INBREED	1	0.8276	14.03**	1	0.5362	5.03*
POP	1	0.0027	0.07	1	0.0015	0.07
FAM(POP)	22	0.0416	0.92	9	0.0227	0.45
COMP	1	0.7307	16.09***	1	1.2197	23.98***
INBREED X FAM(POP)	18	0.0590	1.3	10	0.1065	2.09*
INBREED X COMP	1	0.0013	0.03	1	0.0273	0.54
Error	127	0.0454		97	0.0509	

† **F Ratios.** Unless specified below F ratios calculated as MS_{effect}/MS_{error}

Effect	*F Ratio*
INBREED	$MS_{INBREED}/(MS_{FAMxINBREED})$
POP	MS_{POP}/MS_{FAM} (Female Fecundity and Male Mating only)

Table 3.3: Summary of average (± se) larva-adult survival, total fecundity of surviving females and mating success of surviving males, as well as inbreeding depression (δ) and number of haploid lethal equivalents (β) for these traits. All traits were measured after rearing in either low or high larval competition. Larval-adult survival and male mating success were measured relative to a standard competitor (LCI or MCI) or in absolute terms as the percent survival or the percent of the total matings. Female fecundity is the average number of offspring produced on days 1-8 (early), days 8-16 (late), or both. N is the sample size. Traits were evaluated using the female lineage or the male lineage.

*** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$

FITNESS FOR DIFFERENT LEVELS OF LARVAL COMPETITION

Competition	OUTBRED		INBRED		δ		β	
Larval-Adult Survival								
LOW	*Maternal*	*Paternal*	*Maternal*	*Paternal*	*Maternal*	*Paternal*	*Maternal*	*Paternal*
AVG LCI	1.84 ± 0.02	1.84 ± 0.02	1.45 ± 0.02	1.46 ± 0.03	0.23 ± 0.03***	0.20 ± 0.02***	0.95	0.90
AVG % Survival	89.36 ± 0.80	88.97 ± 0.64	72.24 ± 1.05	71.98 ± 1.14	0.19 ± 0.01***	0.18 ± 0.07***	0.85	0.84
	N= 151	N= 128	N= 140	N= 118				
HIGH								
AVG LCI	5.15 ± 0.26	4.87 ± 0.27	1.84 ± 0.14	1.77 ± 0.13	0.63 ± 0.03***	0.62 ± 0.03***	4.12	3.89
AVG % Survival	43.38 ± 1.14	42.14 ± 0.81	27.83 ± 1.33	28.53 ± 1.01	0.34 ± 0.04*	0.32 ± 0.18***	1.76	1.52
	N= 156	N=126	N= 134	N=110				
Male Mating Success								
LOW	*Maternal*	*Paternal*	*Maternal*	*Paternal*	*Maternal*	*Paternal*	*Maternal*	*Paternal*
AVG MCI	11.26 ± 0.87	11.53 ± 1.14	5.31 ± 0.61	5.18 ± 0.68	0.57 ± 0.05***	0.48 ± 0.10**	3.01	2.58
AVG % Matings	76.98 ± 2.80	74.74 ± 2.24	58.30 ± 2.80	55.96 ± 3.27	0.26 ± 0.07*	0.24 ± 0.19**	1.22	1.11
	N= 53	N= 31	N= 53	N= 33				
HIGH								
AVG MCI	6.89 ± 0.68	5.35 ± 0.57	3.12 ± 0.43	2.63 ± 0.36	0.51 ± 0.09***	0.45 ± 0.10**	3.17	2.39
AVG % Matings	65.04 ± 2.25	60.50 ± 2.49	45.92 ± 2.87	42.90 ± 2.79	0.32 ± 0.06***	0.28 ± 0.18**	1.61	1.34
	N= 33	N= 24	N= 34	N= 25				
Female Fecundity								
LOW	*Maternal*	*Paternal*	*Maternal*	*Paternal*	*Maternal*	*Paternal*	*Maternal*	*Paternal*
Early	74 ± 1.29	74 ± 1.60	73 ± 1.20	75 ± 1.56	-0.04 ± 0.05	-0.05 ± 0.06	0	0
Late	83 ± 1.24	83 ± 1.52	58 ± 1.62	55 ± 2.04	0.22 ± 0.05***	0.25 ± 0.05***	0.98	1.16
Total # Offspring	157 ± 1.90	157 ± 2.37	131 ± 2.05	128 ± 2.27	0.14 ± 0.03***	0.16 ± 0.04***	0.72	0.71
	N= 292	N= 184	N= 245	N= 168				
HIGH								

Table 3.4: Repeated-measures analysis of variance comparing the fitness loss due to sib mating for the average number of offspring produced by females on days 1-8 (early fecundity) and days 8-16 (late fecundity) under different levels of competition. TIME represents the early and late measures of fecundity. *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$.

	Maternal Lineage			Paternal Lineage		
Source	*dfNum*	*dfDen*	*F*	*dfNum*	*dfDen*	*F*
INBREED	1	1319	0.05	1	1053	4.2*
COMP	1	1319	3.17	1	1053	2.42
POP	1	1319	0.85	1	1053	0.02
FAM(POP)	27	1319	3.10***	15	1053	4.46***
INBREED x FAM(POP)	27	1319	3.87***	16	1053	4.69***
INBREED x COMP	1	1319	0.01	1	1053	2.17
COMP x FAM(POP)	23	1319	5.58***	12	1053	9.34***
INBREED x COMP x FAM(POP)	13	1319	1.43	7	1053	2.14*
TIME	1	1319	62.29***	1	1053	100.74***
INBREED x TIME	1	1319	69.36***	1	1053	106.36***
TIME x COMP	1	1319	28.19***	1	1053	19.75***
TIME x FAM(POP)	27	1319	2.46***	15	1053	3.17***
TIME x INBREED x COMP	1	1319	4.61*	1	1053	15.57***
TIME x COMP x FAM(POP)	22	1319	5.04***	11	1053	9.11***
TIME x INBREED X POP x COMP	2	1319	0.91	2	1053	11.11***
TIME x INBREED x COMP x FAM(POP)	38	1319	2.75***	21	1053	3.84***

Table 3.5: Cumulative fitness loss in male and female inbred offspring and from sib mating using both maternal and paternal lineage analyses. A) Summary of average (± se) inbreeding depression (δ) and number of haploid lethal equivalents (β), where N equals the number of families. The overall fitness loss from sib mating is the average of the fitness loss from sons and daughters. B) Analysis of variance for cumulative offspring fitness. The model included male vs. female (SEX), high vs. low competition (COMP), population Gala vs. Mayo (POP), and family (FAM). *** $p < 0.001$, ** $p < 0.01$ * $p < 0.05$

A. SUMMARY OF CUMULATIVE FITNESS

Competition	δ		β	
	Maternal	*Paternal*	*Maternal*	*Paternal*
Female Cumulative Fitness				
LOW	0.33 ± 0.03 N=26	0.36 ± 0.03 N= 14	1.60	1.80
HIGH	0.66 ± 0.04 N=15	0.63 ± 0.06 N=7	4.19	3.91
Male Cumulative Fitness				
LOW	0.67 ± 0.04 N=11	0.64 ± 0.06 N=8	4.43	4.07
HIGH	0.81 ± 0.04 N=11	0.76 ± 0.07 N=7	6.64	5.71
Cumulative Fitness Loss from Sib Mating				
LOW	0.50 ± 0.04	0.50 ± 0.05	2.77	2.77
HIGH	0.73 ± 0.04	0.70 ± 0.07	5.24	4.82

B. ANOVA CUMULATIVE FITNESS

		Maternal Analysis			**Paternal Analysis**	
Source	*df*	*MS*	*F*†	*df*	*MS*	*F*†
COMP	1	0.3768	11.18*	1	0.1773	6.54
SEX	1	0.3185	40.83***	1	0.1987	10.86**
POP	1	0.0213	2.73	1	0.0485	2.65
FAM(POP)	7	0.0286	3.66*	5	0.0316	1.73
SEX x COMP	1	0.0117	1.51	1	0.0419	2.29
FAM(POP) x COMP	8	0.0337	4.32**	4	0.0732	4.00*
Error	16	0.0078		10	0.0182	

†**F Ratios.** Unless specified below F ratios calculated as MS_{effect}/MS_{error}

Effect	*F Ratio*
COMP	$MS_{COMP}/(MS_{FAMxCOMP})$

Table 3.6: Summary of the effects of inbreeding on individual fitness components in *Drosophila melanogaster* measured under benign laboratory conditions. The number of haploid lethal equivalents (β) was calculated as described in the text ($\beta = -[\ln(w_{inbred} / w_{outbred})]/F$), given a single level of inbreeding (F) or as the slope of the regression of Ln(fitness) on F. For chromosome homozygotes β was calculated according to Supplementary Table 3.1. For fecundity measures the age of adult females is the age range over which females laid eggs and for mating success it is the age at which males were tested. Hatchability and larval survival for both inbred (IB) and outbred (OB) individuals is included when the data was available. All values for this study are from the maternal analysis and include both the competitive index and percent success (in parenthesis) for larval survival and male mating. Cumulative fitness for each sex is a multiplicative measure that was calculated across the same life history stages for each study (see Methods).

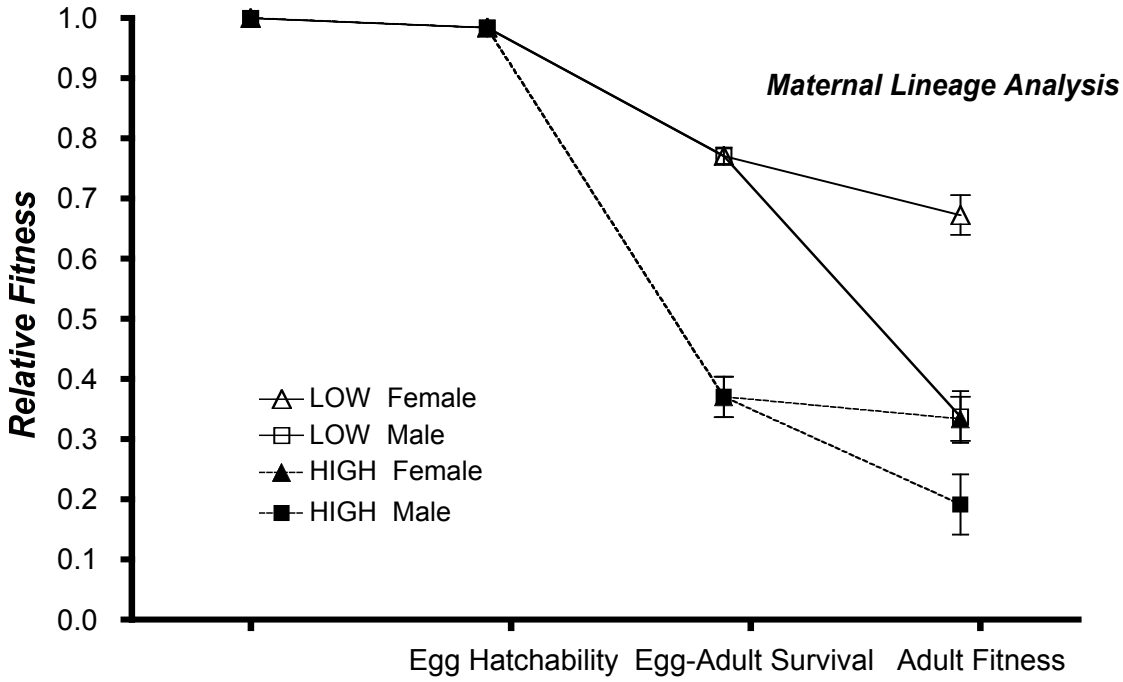
* Fitness measure is relative to a standard competitor (i.e. MCI or LCI).

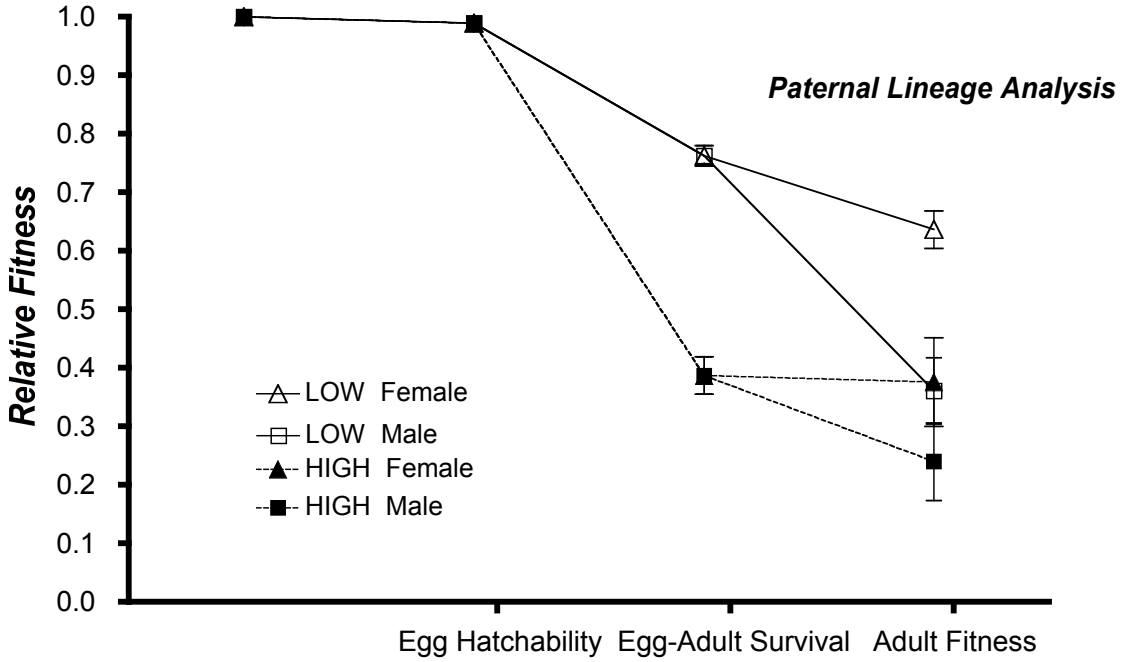
† Male fitness used to calculate cumulative fitness was the number of progeny a male produced when mated to a stock female.

‡ Male fitness used to calculate cumulative fitness was the male competitive index (MCI).

Life History Stage	F	Type of Inbreeding			β	Source
Egg Hatchability			**%OB**	**%IB**		
	0.25	full-sib mating	98.6	96.8	0.06	This study
	0.25	full-sib mating	96.0	90.0	0.26	Biémont 1978
Egg-Adult Survival			**%OB**	**%IB**		
egg-pupa survival	0.25-0.73	full-sib mating	80.0	60.0	0.43	Garcia et al 1994
larval-adult survival	0.25	full-sib mating	89.4	72.2	0.95* (0.85)	This study
	0.25	full sib mating	85.6	66.7	1.00	Ehiobu et al 1989
	0.25-0.75	full sib mating	61.9	49.9	0.37	Tantawy 1957
	0.37	chrom 2 homozygotes	NA	NA	0.30	Miller and Hedrick 1993
egg-adult survival	0.25-0.88	full sib mating	NA	NA	2.67*	Latter and Roberston 1962
	0.25	full-sib mating	90.0	70.0	1.01	Biémont 1978
	0.25	full-sib mating	80.0	72.0	0.42	Robinson et al 2009
	0.37	chrom 2 homozygotes	NA	NA	0.82	Bijlsma et al 1999
	0.65	chrom 3 homozygotes	NA	NA	0.89	Mackay 1985
Adult Female Fitness			**Age**			
female fecundity	0.25	full-sib mating	*6-14 days*		0.00	This study
	0.25		*15-22 days*		0.98	
	0.25	full-sib mating	*3-6 days*		1.10	Ehiobu et al 1989
	0.25	full-sib mating	*7-8 days*		0.57	Robinson et al 2009
	0.50	full-sib mating	*2-5 days*		1.12	Dahlgaard and Hoffmann 2000
	0.50	full-sib mating	*1-5 days*		0.30	Miller 1994
	0.55	chrom 2 homozygotes	*1-4 days*		0.00	Miller and Hedrick 1993
	0.98	chrom2,3 homozygotes	*1-7 days*		0.34	Hughes et al 2002
			8-14 days		0.77	
			15-21 days		0.71	
	0.65	chrom 3 homozygotes	unknown		1.25	Mackay 1985
Adult Male Fitness			**Age**			
male mating success	0.25	full-sib mating	*14-18 days*		3.01* (1.22)	This study
	0.25-0.98	full-sib mating	*4-7 days*		0.81*	Sharp 1984
	0.25	full-sib mating	*5 days*		5.39	Pendlebury and Kidwell 1974
	0.25	full-sib mating	*5 days*		2.25	
	0.37	chrom 2 homozygotes	*3 days*		1.02	Kosuda 1983
	0.37	chrom 2 homozygotes	*3 days*		3.49	Miller and Hedrick 1993
	0.37	chrom 2 homozygotes	*2 days*		1.86*	Brittnacher 1981
	0.44	chrom 3 homozygotes	*3 days*		0.43*	Hughes 1995
		chrom 3 homozygotes	*21 days*		1.14*	
	0.44	chrom 3 homozygotes	*3 days*		1.04*	Partridge et al 1985
Cumulative Fitness			**Sex**			
	0.25	full-sib mating	female		1.60* (1.50)	This study
			male		4.25* (2.15)	
	0.25	full-sib mating	female		0.91	Robinson et al 2009
			male		0.92†	
	0.37	chrom 2 homozygotes	female		0.27	Miller and Hedrick 1993
			male		3.79‡	
	0.65	chrom 3 homozygotes	female		2.13	Mackay 1985

Figure 3.1: Cumulative inbreeding depression in male and female offspring resulting from sib mating. Two levels of larval competition are shown: low and high. Cumulative relative fitness (Winbred / Woutbred) averaged across families (± SE) is plotted at each life history stage from egg to adult. The adult fitness measure is fecundity (females) or mating success (males).


Maternal Lineage Analysis
Relative Fitness
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
LOW Female
LOW Male
HIGH Female
HIGH Male
Egg Hatchability
Egg-Adult Survival
Adult Fitness


Paternal Lineage Analysis
Relative Fitness
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
LOW Female
LOW Male
HIGH Female
HIGH Male
Egg Hatchability
Egg-Adult Survival
Adult Fitness

Appendix: Calculation of level of inbreeding (F) in chromosome homozygote studies in Table 3.6.

Based on the published genome sequence of *D. melanogaster* (Adams *et al.*, 2000), when chromosomes II and III are made homozygous this corresponds to F = 1 for roughly 0.37 and 0.44 of the autosomal genome respectively. For traits expressed in both sexes or only in males the sex chromosomes are not expected to contribute to overall levels of inbreeding depression due to the removal/purging of recessive deleterious alleles in males (e.g. egg-adult viability, Eanes et al. (1985)). Therefore, we defined F = 0.37 and F = 0.44 for chromosome II and III homozygous respectively for egg-adult viability and male fitness measure. These values are the minimum level of inbreeding (F) expected when each chromosome is made homozygous. To account for sex-limited genetic load expressed on the X-chromosome for female fertility (Tracy & Ayala, 1974; Eanes *et al.*, 1985), we adjusted the level of expected inbreeding (F) for female fecundity (increased 18%) to include the X chromosome being totally inbred. During the creation of lines homozygous for single chromosomes the remaining portion of the genome is expected to become randomly inbred as a result. Mackay (1985) adjusted for this in the estimation of the level of inbreeding (F), and therefore the calculated F = 0.65 was used in Table 3.6.

Concluding Remarks

Environmental stress is a central focus of research across many biological disciplines. Consequently, it is well established that environmental stress influences cellular processes, individual physiology, genetic variation, and the process of natural selection (Hoffmann & Parsons, 1991; Loeschcke *et al.*, 2004). From a conservation standpoint it is becoming increasingly important that biologists understand how inbreeding induced by small population size may result in greater susceptibility to the effects of environmental stress and therefore an elevated extinction risk. My dissertation focuses on understanding the relationship between environmental stress and inbreeding, specifically what factors contribute to the variation in inbreeding depression observed under stressful conditions.

The first chapter of my dissertation demonstrated a strong positive linear relationship between stress level and the magnitude of inbreeding depression, both in laboratory populations of *Drosophila* and for populations experiencing fluctuating field conditions. This is the first study to demonstrate that variation in levels of inbreeding depression in the wild are correlated to changes in stress levels experienced during seasonal extremes. This work highlights the need for researchers to measure and report stress levels when examining the effects of different types of stress on the expression of inbreeding depression. Studies that find stress does not amplify inbreeding depression are misleading without information on stress levels and may actually misidentify which conditions are by

definition stressful (Chen, 1993; Pray *et al.*, 1994). Finally, the underlying molecular and biochemical mechanisms that are responsible for the general linear relationship between inbreeding depression and stress level are unknown. Future studies should focus on determining the genetic basis of inbreeding depression under stress using techniques such as microarray and QTL analysis.

The second chapter of my dissertation demonstrated that stress type and timing of exposure to stress are important determinants of variation in levels of inbreeding depression expressed under stress. This is the first study to standardize stress level and thus directly compare the effects of several types of abiotic and biotic stress on the magnitude of inbreeding depression expressed at several life history stages (during exposure and post exposure). Specifically, I found that not all stresses amplified inbreeding depression during exposure (larval survival) despite causing the same level of mortality in outbred individuals (equivalent stress levels). In addition, only the two biotic stresses were found to purge genetic load and reduce inbreeding depression for post exposure male mating success. This suggests that these stresses affect genes and/or pathways that are important for larval development and survival as well as mating success as adult males. Future challenges include identifying those stresses that effectively purge genetic load and elucidating the mechanisms by which this can occur. In addition, studies are needed that explore the genetic basis of inbreeding depression under stress. Future work should focus on determining if variation in

levels of inbreeding depression under stress can be explained by differences in the number of genes or pathways affected by the type of environmental stressor.

The third chapter of my dissertation I examined how both sexual selection and environmental stress affects the magnitude of inbreeding depression expressed in inbred males verses inbred females. It has been hypothesized that sexual selection, via female choice and/or male competitive interactions, increases selection against deleterious alleles expressed in homozygous inbred males (Jarzebowska & Radwan, 2009; Whitlock & Agrawal, 2009), leading to higher inbreeding depression in male reproductive fitness. My findings support this hypothesis, with males suffering a two-fold higher cost to being inbred than females. This is presumably due to the inclusion of both female choice and/or male competition in determining the mating success of males, while female fecundity does not include such elements of sexual selection. However, we found no significant correlation between inbreeding depression in male reproduction and other traits. Future work is needed to determine if sexual selection can in general alleviate mutational load in populations and whether this is an important phenomenon in natural populations.

The results of my dissertation raise many additional questions regarding the relationship between inbreeding and environmental stress. Can biologists identify the types of stress or times of the year that impose the greatest extinction risk for small inbred populations in the wild? Future studies should strive to understand not only the short-term effects of stress on levels of inbreeding

depression but also the long-term evolutionary implications of interactions between inbreeding and stress. Can exposure to stress purge harmful genetic load and enable populations to better survive environmental challenges? Information on inbreeding-stress interactions also needs to be incorporated into population viability analyses commonly applied in conservation biology.

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