Extreme temperatures increase the deleterious consequences of inbreeding under laboratory and semi-natural conditions

Torsten N. Kristensen^{1,2,*}, J. Stuart F. Barker³, Kamilla S. Pedersen^{1,2} and Volker Loeschcke²

¹Department of Genetics and Biotechnology, University of Aarhus, Blichers Allé 20, 8830 Tjele, Denmark

²Aarhus Centre for Environmental Stress Research, Department of Biological Sciences, University of Aarhus, Ny Munkegade, Build. 1540, 8000 Aarhus C, Denmark

³School of Environmental and Rural Science, University of New England, Armidale, NSW 2351, Australia

The majority of experimental studies of the effects of population bottlenecks on fitness are performed under laboratory conditions, which do not account for the environmental complexity that populations face in nature. In this study, we test inbreeding depression in multiple replicates of inbred when compared with non-inbred lines of *Drosophila melanogaster* under different temperature conditions. Egg-to-adult viability, developmental time and sex ratio of emerging adults are studied under low, intermediate and high temperatures under laboratory as well as semi-natural conditions. The results show inbreeding depression for egg-to-adult viability. The level of inbreeding depression is highly dependent on test temperature and is observed only at low and high temperatures. Inbreeding did not affect the developmental time or the sex ratio of emerging adults. However, temperature affected the sex ratio with more females relative to males emerging at low temperatures, suggesting that selection against males in pre-adult life stages is stronger at low temperatures. The coefficient of variation (CV) of egg-to-adult viability within and among lines is higher for inbred flies and generally increases at stressful temperatures. Our results contribute to knowledge on the environmental dependency of inbreeding under different environmental conditions and emphasize that climate change may impact negatively on fitness through synergistic interactions with the genotype.

Keywords: inbreeding × environment interactions; inbreeding depression; temperature stress; phenotypic variance; semi-natural environments

1. INTRODUCTION

A decline in population size is often accompanied by loss of genetic variability and inbreeding depression, which in turn may impact on the extinction risk of threatened populations and the evolution of inbreeding avoidance behaviour and physiological responses (Crnokrak & Roff 1999; Hedrick & Kalinowski 2000; Amos & Balmford 2001; Keller & Waller 2002; Spielman *et al.* 2004; Kristensen *et al.* 2006; O'Grady *et al.* 2006). Apart from genetic concerns, environmental stress induced by, for example, climate change and pollution imposes direct threats to the persistence of populations (World Conservation Monitoring Centre 1992; Hoffmann & Parsons 1997; Baillie *et al.* 2004).

The relative contributions of genetic versus ecological factors to extinction probabilities in wild populations are still being debated (Caro & Laurenson 1994; Caughley 1994; Frankham 2005; Jamieson *et al.* 2006).

The deleterious consequences of inbreeding depend on the genetic load carried by a population and may be trait and environment dependent (Armbruster & Reed 2005; Szulkin & Sheldon 2007; Kristensen *et al.* 2008a).

A number of recent studies have suggested that the deleterious consequences of inbreeding are reinforced under harsh environmental conditions (Keller *et al.* 2002; Reed *et al.* 2003, 2007; Da Silva *et al.* 2006), and that studies investigating inbreeding effects under laboratory conditions that are often benign may not be representative for consequences of inbreeding in variable natural environments (Chen 1993; Pray *et al.* 1994; Kristensen *et al.* 2008a). On the other hand, studies of the effects of inbreeding and interactions between inbreeding and the environment in natural populations often suffer from low replication, incomplete pedigree information, lack of proper controls and the fact that the presumed neutral molecular markers often used may not reflect levels of detrimental and adaptive genetic variation. Therefore, most individual field studies lack sufficient statistical power to uncover detailed patterns of inbreeding effects in nature (Szulkin & Sheldon 2007). Experiments performed under semi-natural conditions with replicated populations with known levels of inbreeding may therefore be a useful supplement for increasing our knowledge of the importance of inbreeding × environment interactions on fitness.

Temperature is one of the most important variables that determine distribution and abundance of species (Cossins & Bowler 1987; Hoffmann & Parsons 1997).

* Author and address for correspondence: Department of Genetics and Biotechnology, University of Aarhus, Blichers Allé 20, 8830 Tjele, Denmark (torsten.nygaard@agrsci.dk).

The mean global temperature is increasing at an unprecedented rate and weather conditions are becoming more unpredictable, increasing the frequency of more extreme temperatures (IPCC 2007). This poses great challenges to all organisms and is one factor why distribution maps are currently changing and population sizes decreasing for many species (Perry *et al.* 2005; Walther *et al.* 2005; IPCC 2007). Therefore, potential effects of interactions between genotype and temperature on fitness should be investigated with respect to their impact on extinction risk and hence biodiversity.

Most published studies on responses to extreme temperatures and the importance of interactions between genotypes and thermal conditions have been performed in the laboratory at constant low or high temperatures (Bijlsma *et al.* 1999; Dahlggaard & Hoffmann 2000; Kristensen *et al.* 2003). They therefore do not take into account the potential importance of daily and seasonal fluctuations in temperature, humidity and light intensity. Thus, the ecological relevance of such studies can be questioned (Gibbs 1999; Harshman & Hoffmann 2000; Kristensen *et al.* 2007, 2008a,b). Therefore, there is a need to study how inbreeding affects the mean and variance of fitness-related traits in highly replicated experiments and under different ecologically relevant climatic conditions. Such studies are of crucial importance for evaluating the impact of climate change on biodiversity and for designing sustainable conservation strategies for small and isolated populations.

In this highly replicated study with the model organism *Drosophila melanogaster*, we test the hypothesis that the level of inbreeding depression is dependent on climatic conditions. Egg-to-adult viability, developmental time and sex ratio of emerging adults are tested in eight inbred and eight non-inbred control lines under low, intermediate and high temperatures, both under laboratory and semi-natural conditions. By testing flies under natural temperature, humidity and daylight conditions, we attempted to move beyond the limitations of laboratory investigations of inbreeding $\times$ temperature interactions. The results reveal strong interactions between breeding regime (inbred versus non-inbred) and temperature for egg-to-adult viability and developmental time, with inbreeding depression observed only for egg-to-adult viability at low and high temperatures, and not at presumably more benign intermediate temperatures.

2. MATERIAL AND METHODS

(a) Inbreeding procedure and maintenance of the lines in the laboratory

Inbred and non-inbred control lines were generated from a genetically diverse mass population of *D. melanogaster* (see Bublly & Loeschcke 2005 for details). Eight independent lines with expected equivalent levels of inbreeding ($F \approx 0.67$) were obtained through five generations of full-sib mating (see Kristensen *et al.* 2005 and Pedersen *et al.* 2005 for details on the inbreeding procedure and maintenance of stocks). After reaching the desired level of inbreeding, all lines were flushed to a minimum of 500 individuals (within two generations). Eight non-inbred lines, each founded by approximately 500 breeding individuals, were established at the time when inbreeding was initiated. The non-inbred and flushed inbred lines were thereafter held at high N_e ($N > 1000$). Throughout

and following the inbreeding procedure, all flies were maintained in a climate room ($25 \pm 0.2^\circ\text{C}$, 40–60% relative humidity, 12 L : 12 D cycle). The lines used in this study were established in 2004 and kept at the University of Aarhus, Denmark, for approximately 75 generations before the present experiments were carried out.

(b) Experiments on inbreeding effects under laboratory and semi-natural conditions

Eight inbred and eight non-inbred control lines were tested for egg-to-adult viability, developmental time and sex ratio under laboratory and semi-natural conditions at low, intermediate and high temperatures. All experiments were performed in the same generation. Twenty eggs were collected into each of 10 vials per line, location (laboratory and semi-natural) and temperature treatment (low, intermediate and high). The total number of eggs and vials used were, respectively, 19 200 and 960. All tests in the laboratory were performed in incubators at the University of Aarhus, Denmark, whereas tests under semi-natural conditions were performed in Denmark (low temperatures) or Queensland, Australia (intermediate and high temperatures). Two generations before the experiments were performed, we sent developing larvae from all 16 lines to Australia. These were maintained at the University of Queensland for two generations at standard laboratory conditions prior to collection of the eggs that were used in the experiments. All eggs were collected in the laboratory at 25°C at the University of Aarhus, Denmark, or the University of Queensland, Australia. Thereafter, the vials were randomized and transferred to incubators in the laboratory or to a field site (woodland) within 2–3 hours after being collected. In the field, the vials with eggs from all lines were randomized in racks and placed approximately 1.5 m above ground hanging from a tree and shaded from direct sunlight. Temperatures in the incubators were 5.5°C at night (01.00–06.00) and 14.5°C during the day (06.00–01.00) for the low temperature treatment, constant 25°C for the intermediate temperature treatment, and 20°C at night (20.00–08.00) and 33.5°C during the day (08.00–20.00) for the high temperature treatment. At the intermediate temperature treatments (25°C), the light was on from 08.00 to 20.00 (12 L : 12 D cycles), whereas in the other treatments the light was on during 'day' temperatures (19 L : 5 D and 12 L : 12 D cycles for the low and high temperature treatments, respectively). Mean temperatures throughout development at low, intermediate and high temperatures were, respectively, 12.6, 25 and 26.8°C under laboratory conditions, and 12.4, 21.6 and 24.6°C under semi-natural conditions in the field (figure 1).

Egg-to-adult viability was estimated as the proportion of eggs, which survive to adulthood in each vial, out of the initial 20 eggs per vial; developmental time as the time taken from egg laying to emergence of adults (for adults that actually emerge from pupae—counted daily) and the sex ratio as the number of males emerging relative to the total number of flies emerging. All traits were investigated at both localities and for all three temperature regimes.

(c) Analyses

Nested ANOVAs were used to test the effects of location (laboratory or semi-natural), temperature (low, intermediate or high) and breeding regime (inbred or non-inbred) on egg-to-adult viability, developmental time and sex ratio. Males and females were pooled for ease of interpretation of

Figure 1. Temperatures in the semi-natural environments during the experimental periods for low (grey curve), intermediate (light grey curve) and high (black curve) temperatures. Development at low temperatures lasted for up to 50 days and therefore temperature registrations also lasted this long.

the results for the traits egg-to-adult viability and developmental time (analyses of the sexes treated separately did not lead to different conclusions, results not shown). Line was nested within breeding regime as a random factor. In the full factorial nested ANOVAs, the error degrees of freedom were 913 for developmental time and sex ratio, whereas the error degrees of freedom were 934 for egg-to-adult viability. The difference is due to the fact that some vials did not produce any emerging adults. Differences among inbred and non-inbred lines for all three traits were tested separately for each location/temperature regime by nested ANOVAs with line nested within breeding regime as a random factor and with breeding regime as a fixed factor. Significances were adjusted according to the sequential Bonferroni correction.

Within line CVs were estimated for egg-to-adult viability (CVs are only presented for this trait as inbreeding depression was not observed for the other two traits investigated) across the 10 replicate vials per line at each location and temperature. In total, 96 within line CV estimates were obtained (2 locations $\times$ 3 temperatures $\times$ 16 lines (8 inbred and 8 outbred)). CVs were also calculated across lines within each location and temperature. Average egg-to-adult viability estimates were calculated for all locations, temperatures and lines, and the CVs were obtained based on these line means. In total, 12 across line CV estimates were obtained (2 locations $\times$ 3 temperatures $\times$ 2 breeding regimes). ANOVA models were used to test differences in within and across line CVs, with location, temperature and breeding regime as fixed factors.

3. RESULTS

(a) Egg-to-adult viability

Location, temperature and breeding regime all had a significant effect on egg-to-adult viability (location: $F_{1,934} = 7.24$, $p < 0.01$; temperature: $F_{2,934} = 293.59$, $p < 0.0001$; breeding regime: $F_{1,934} = 23.93$, $p < 0.001$). Egg-to-adult viability was higher at intermediate temperatures and generally lowest at low temperatures (figure 2). Egg-to-adult viability was higher under semi-natural relative to laboratory conditions at high temperatures (figure 2). Across locations and temperatures, viability was 22.5% lower in inbred relative to non-inbred flies

Figure 2. Proportion egg-to-adult viability $\pm$ s.e. for development at low, intermediate and high temperatures under laboratory and semi-natural conditions. Significant differences in egg-to-adult viability between inbred (black bars) and non-inbred (grey bars) lines at different temperatures are indicated (***) $p < 0.001$; table 1).

(figure 2). The location $\times$ temperature interaction was significant ($F_{2,934} = 95.21$, $p < 0.0001$) showing that temperature impacted egg-to-adult viability differently under laboratory when compared with semi-natural conditions. This interaction may be due to the different temperature regimes across the two locations. The interaction between temperature and breeding regime was highly significant ($F_{2,934} = 27.81$, $p < 0.0001$), showing that the level of inbreeding depression depends upon the environmental conditions and is more severe at low and high temperatures relative to intermediate temperatures (figure 2). The effect of inbreeding on egg-to-adult viability did not differ between laboratory and semi-natural conditions ($F_{1,934} = 0.04$, n.s.), but the three-way interaction between location, temperature and breeding regime was significant ($F_{2,934} = 3.88$, $p < 0.05$). Lines differed significantly in egg-to-adult viability ($F_{14,934} = 13.71$, $p < 0.0001$). The *post hoc* analyses testing for differences in egg-to-adult viability in inbred and outbred lines at the two locations and for the three temperature treatments showed that significant inbreeding depression was only observed at low temperatures under laboratory and semi-natural conditions, and at high temperatures under laboratory conditions (figure 2; table 1). In these tests, line was a significant factor in five of six cases.

(b) Developmental time

The fixed factors location and temperature affected developmental time significantly, whereas breeding regime did not impact on developmental time (location: $F_{1,913} = 43.35$, $p < 0.0001$; temperature: $F_{2,913} = 28607.50$, $p < 0.0001$; breeding regime: $F_{1,913} = 1.92$, n.s.). Developmental time was almost four times longer at low temperatures, both under laboratory and semi-natural conditions, when compared with intermediate or high temperatures, slightly longer at intermediate temperatures in semi-natural when compared with laboratory conditions, and slightly longer at high temperatures under laboratory conditions when compared with semi-natural conditions (figure 3).

Table 1. Results from nested ANOVAs testing effects of line (d.f. = 14 in all cases) and breeding regime (d.f. = 1) for each location/temperature for the traits egg-to-adult viability, developmental time and sex ratio (error d.f. = 144 in all cases). (Significance adjusted according to the sequential Bonferroni correction. n.s., non-significant; (n.s.), significant before but not after Bonferroni correction. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.)

	egg-to-adult viability		developmental time		sex ratio	
	breeding regime	line	breeding regime	line	breeding regime	line
low laboratory	***	***	(n.s.)	***	n.s.	n.s.
low semi-natural	***	***	n.s.	**	n.s.	(n.s.)
intermediate laboratory	(n.s.)	**	n.s.	***	n.s.	(n.s.)
intermediate semi-natural	n.s.	***	n.s.	*	n.s.	n.s.
high laboratory	***	(n.s.)	n.s.	***	n.s.	n.s.
high semi-natural	n.s.	***	n.s.	n.s.	n.s.	n.s.

Lines differed significantly in developmental time ($F_{14,913} = 2.98$, $p < 0.001$). The interaction between location and temperature was significant ($F_{2,913} = 161.07$, $p < 0.0001$), showing that the impact of temperature on developmental time differed between laboratory and field conditions. This result, however, may be affected by the fact that the low, intermediate and high temperature regimes were not identical under laboratory and semi-natural conditions. The interaction between breeding regime and temperature was highly significant ($F_{2,913} = 8.58$, $p < 0.001$), showing that inbreeding effects on developmental time depend on the temperature conditions. This interaction was primarily caused by slower development for inbred lines at low temperatures in the laboratory, a tendency in the opposite direction at high temperatures in the laboratory, and no difference between developmental time for inbred and outbred lines at the remaining locations/temperatures (figure 3). The interaction between location and breeding regime was not significant ($F_{1,913} = 2.94$, n.s.), but the three-way interaction between location, temperature and breeding regime was significant ($F_{2,913} = 11.89$, $p < 0.0001$). Even though the overall test showed no effect of breeding regime, we tested for inbreeding depression and line effects in the two locations and for the three temperature treatments. After Bonferroni correction, developmental time was not significantly different among breeding regimes in any of the comparisons, but significant line effects were observed in some comparisons (table 1).

(c) Sex ratio

The sex ratio was significantly affected by temperature ($F_{2,913} = 9.26$, $p < 0.0001$). This effect was mainly due to the lower sex ratio (a higher number of females emerging) at low temperatures (figure 4). At intermediate and high temperatures, 50.3 and 50.0% of the emerging flies were males, whereas at low temperatures on average across breeding regimes and localities only 44.5% of the emerging flies were males. None of the other fixed effects, interactions among them, nor line significantly affected the sex ratio (location: $F_{1,913} = 2.71$, n.s.; breeding regime: $F_{1,913} = 1.76$, n.s.; location $\times$ temperature: $F_{2,913} = 1.72$, n.s.; temperature $\times$ breeding regime: $F_{2,913} = 0.46$, n.s.; location $\times$ breeding regime: $F_{1,913} = 1.47$, n.s.; location $\times$ temperature $\times$ breeding regime: $F_{2,913} = 0.20$, n.s.; line: $F_{14,913} = 0.76$, n.s.). Neither breeding regime nor line significantly affected sex ratio when tested for the separate locations/temperatures (table 1).

Figure 3. Developmental time in days $\pm$ s.e. for development at low, intermediate and high temperatures under laboratory and semi-natural conditions. Grey bars, non-inbred; black bars, inbred.

(d) The effects of location, temperature and inbreeding on the coefficient of variation in egg-to-adult viability

CVs based on egg-to-adult viability in the 10 replicate vials per line were calculated for all lines, locations and temperatures. Average values are presented in table 2. ANOVA results show that location, temperature and breeding regime all had significant effects on the within line CVs (location: $F_{1,84} = 4.01$, $p < 0.05$; temperature: $F_{2,84} = 9.51$, $p < 0.001$; breeding regime: $F_{1,84} = 11.46$, $p < 0.01$). The CVs were on average higher for the inbred lines relative to the outbred control lines and higher at low and high temperatures relative to intermediate temperatures. The interaction between location and temperature was significant, whereas none of the other interaction terms affected the CVs (location $\times$ temperature: $F_{2,84} = 11.46$, $p < 0.01$; temperature $\times$ breeding regime: $F_{2,84} = 1.79$, n.s.; location $\times$ breeding regime: $F_{1,84} = 0.67$, n.s.; location $\times$ temperature $\times$ breeding regime: $F_{2,84} = 1.01$, n.s.).

To get an estimate of the variation across lines, egg-to-adult viability estimates were calculated for all lines in the respective locations and temperatures. The CVs were calculated on the basis of these line means (table 2). Results show that inbreeding increased CVs (breeding regime: $F_{1,12} = 20.70$, $p < 0.01$), whereas neither location

Figure 4. Sex ratio $\pm$ s.e. (defined as the number of male flies emerging relative to the total number of emerging adults) for development at low, intermediate and high temperatures under laboratory and semi-natural conditions. Grey bars, non-inbred; black bars, inbred.

Table 2. CVs for egg-to-adult viability. The estimates are the average CVs for the eight lines (mean within line CVs) and CVs of the across line means (across line CVs) for all temperatures, locations and breeding regimes.

temperature/ location	breeding regime	mean within line CVs	across line CVs
low laboratory	control	21.73	17.05
	inbred	67.46	53.61
low semi-natural	control	27.60	11.16
	inbred	48.93	42.89
intermediate laboratory	control	11.42	2.91
	inbred	12.68	8.63
intermediate semi-natural	control	16.51	13.72
	inbred	30.83	46.78
high laboratory	control	53.50	7.77
	inbred	78.00	47.33
high semi-natural	control	22.45	14.58
	inbred	30.38	49.14

nor temperature impacted on the estimates (location: $F_{1,12} = 1.06$, n.s.; temperature: $F_{2,12} = 1.58$, n.s.).

4. DISCUSSION

The major hypothesis that we set out to test in this study was whether the consequences of inbreeding on fitness components were temperature dependent. The answer to this question is affirmative for the traits egg-to-adult viability and developmental time. For egg-to-adult viability, inbreeding depression was observed only at stressful low and high temperatures (figure 2), whereas the interaction between inbreeding and temperature on developmental time was mainly due to a non-significant trend towards longer development for inbred flies under cold conditions in the laboratory. No effects of inbreeding or inbreeding $\times$ temperature interaction were detected for the sex ratio, a trait expected not to be closely linked to fitness.

This result is very much in accordance with recent studies showing that inbreeding depression may be more

severe under harsh environmental conditions for traits closely linked to fitness (Keller *et al.* 2002; Armbruster & Reed 2005; Da Silva *et al.* 2006; Reed *et al.* 2007; Szulkin & Sheldon 2007). However, our results also show that consequences of inbreeding are highly line and trait specific and emphasize that studies on inbreeding depression should be performed under different environmental conditions, be highly replicated and several life-history traits should be investigated in order to get detailed knowledge on the genetic load of any specific population.

Given the high absolute level of inbreeding of the inbred lines investigated in this study, we were surprised to see that only relatively low levels of inbreeding depression were observed. This may be explained by the history of the investigated lines. They have been held in the laboratory for 75 generations after the bottleneck events. Deleterious alleles may have been purged and mutations may have introduced new variation during that time. However, despite these circumstances, we observed strong interactions between inbreeding and temperature on the fitness traits egg-to-adult viability and developmental time. This indicates that purging of deleterious alleles is environment specific and therefore may not be of great use in conservation practice. Inbreeding levels approaching 0.7 are probably rare in nature (Crnokrak & Roff 1999; Szulkin & Sheldon 2007), but qualitatively we expect the results presented in this paper to be representative for inbreeding $\times$ environment interactions involving less inbred individuals.

Interestingly, deleterious consequences of inbreeding were mainly observed in the laboratory at low and high temperatures. At low temperatures in the laboratory, inbreeding depression was observed for the trait egg-to-adult viability and a non-significant trend towards longer developmental time for inbred relative to non-inbred flies and a skewed sex ratio among the two breeding regimes were observed. At high temperatures under laboratory conditions, inbreeding depression for egg-to-adult viability was observed, whereas no significant inbreeding depression was observed at high temperatures under semi-natural conditions for this trait. This trend towards increased inbreeding depression at low and high temperatures under laboratory conditions relative to at low and high temperatures under semi-natural conditions could be caused by locality-specific variation in the temperature regimes. Under laboratory conditions, the mean low and high temperatures were 12.6 and 26.8°C, respectively, whereas these were 12.4 and 24.6°C, respectively, under semi-natural conditions. However, the main difference between the laboratory and semi-natural temperature regimes was not the absolute levels, but that under semi-natural conditions temperatures decreased gradually in late afternoons and nights and increased gradually in the mornings, whereas under laboratory conditions the temperature shifts were sudden and fast (within 30 min). Under semi-natural conditions, flies therefore have more chance of acclimating to daily changes in temperatures leading to plastic physiological changes that are known to be important for survival (Hoffmann *et al.* 2003; Chown & Terblanche 2007; Kristensen *et al.* 2008b). The distinct temperature distributions under laboratory and semi-natural conditions could explain the different results obtained at the two locations. The laboratory high temperature conditions

indeed seem to be more stressful relative to the high temperatures investigated under semi-natural conditions, since egg-to-adult viability is much lower in both the inbred and non-inbred flies in the laboratory.

The within and across line CVs in egg-to-adult viability showed that inbreeding as well as temperature significantly impacted on these estimates. The overall conclusions were that within as well as across line CVs increase with inbreeding and that within line CVs tend to be higher when development takes place at sub-optimal (low or high) temperatures (table 2). What do these results tell us? According to quantitative genetic theory, one would expect the within line (additive) genetic variance to decrease and the between line genetic variance to increase with inbreeding (Falconer & MacKay 1996). However, changes in non-genetic variance components in inbred lines make it hard to come up with clear expectations of the consequences of inbreeding on variances at the phenotypic level. Inbreeding may increase the environmental variance, which can be interpreted as increased environmental sensitivity (Fowler & Whitlock 1999; Whitlock & Fowler 1999; Kristensen *et al.* 2005). This could explain the increased CVs observed within the inbred lines in the current study. Also, low and high temperatures lead to increased CVs within lines. This may be caused by increased expression of genetic variation that is hidden under benign temperatures, as suggested by Rutherford & Lindquist (1998). Obviously, this hypothesis needs to be tested in more detail. The increase in within line CVs (and a non-significant trend in the same direction across lines) observed at low and high temperatures is interesting because it has been suggested that inbreeding depression should be more pronounced in environments that increase the variability of fitness traits (Szulkin & Sheldon 2007; Waller *et al.* 2008). As discussed recently by Szulkin & Sheldon (2007), increased variability as a consequence of stressful environmental conditions will make the detection of statistically significant inbreeding depression and interactions between genotypes and environments difficult. Performing replicated experiments with high statistical power under semi-natural conditions may therefore be the best way to increase our understanding of inbreeding depression and inbreeding $\times$ environment interactions.

Interestingly, the sex ratio of emerging flies was skewed towards more females relative to males emerging at low temperatures. It is well known that mated adult female *D. melanogaster* are less cold resistant relative to males (Rako & Hoffmann 2006; Jensen *et al.* 2007). However, Jensen *et al.* (2007) presented data indicating that in pupal stages selection may actually be stronger on males relative to females at low temperatures. The mechanism behind this finding is unknown, but our data indicate that selection on eggs, larvae and/or pupae is stronger in males relative to females at low developmental temperatures. If this is a general phenomenon, the evolutionary significance should be investigated in more detail.

5. CONCLUSION

In the current study, we find that inbreeding effects are trait and temperature dependent, with inbreeding depression observed only at stressful low and high temperatures under laboratory and semi-natural conditions

(only at low temperatures) for the trait egg-to-adult viability. We also show that the selection pressure during development is higher for males relative to females at low temperatures. Furthermore, variances (quantified by CVs) in egg-to-adult viability are higher within and among lines with inbreeding than without inbreeding, and increase at stressful temperatures relative to benign intermediate temperatures. The synergistic interactions between inbreeding and temperature observed in this study are of concern in relation to climatic change and emphasize that (i) climate change may have more severe negative consequences for inbred relative to outbred populations and (ii) small populations exposed to inbreeding should be investigated under a range of ecologically relevant environmental conditions and for several traits to verify whether inbreeding is a threat for their long-term persistence.

We are grateful to Doth Andersen, Janne P. Andersen, Louise T. Jensen, Kristina Kristensen, Cino Pertoldi, Jesper G. Sørensen and Trine S. Thomsen for their help with the laboratory assessments; Kjeld Nygaard Kristensen for help with field work; Mark W. Blows, University of Queensland, for kindly letting us use his laboratory facilities; Steve McKechnie, Monash University, for helping with transfer of flies between continents; three anonymous reviewers for their valuable comments and the Danish Research Council for financial support with centre and frame grants (to V.L.) and a postdoctoral fellowship (to T.N.K.).

REFERENCES

- Amos, W. & Balmford, A. 2001 When does conservation genetics matter? *Heredity* **87**, 257–265. (doi:10.1046/j.1365-2540.2001.00940.x)
- Armbruster, P. & Reed, D. H. 2005 Inbreeding depression in benign and stressful environments. *Heredity* **95**, 235–242. (doi:10.1038/sj.hdy.6800721)
- Baillie, J. E. M., Hilton-Taylor, C. & Stuart, S. N. 2004 *IUCN red list of threatened species. A global species assessment*. Gland, Switzerland; Cambridge, UK: IUCN.
- Bijlsma, R., Bundgaard, J. & Van Putten, W. F. 1999 Environmental dependency of inbreeding depression and purging in *Drosophila melanogaster*. *J. Evol. Biol.* **12**, 1125–1137. (doi:10.1046/j.1420-9101.1999.00113.x)
- Bubliy, O. A. & Loeschcke, V. 2005 Correlated responses to selection for stress resistance and longevity in a laboratory population of *Drosophila melanogaster*. *Evol. Biol.* **18**, 789–803. (doi:10.1111/j.1420-9101.2005.00928.x)
- Caro, T. M. & Laurenson, M. K. 1994 Ecological and genetic factors in conservation: a cautionary tale. *Science* **263**, 485–486. (doi:10.1126/science.8290956)
- Caughley, G. 1994 Directions in conservation biology. *J. Anim. Ecol.* **63**, 215–244. (doi:10.2307/5542)
- Chen, X. 1993 Comparison of inbreeding and outbreeding in hermaphroditic *Arianta arbustorum* (L.) (land snail). *Heredity* **71**, 456–461. (doi:10.1038/hdy.1993.163)
- Chown, S. L. & Terblanche, J. S. 2007 Physiological diversity in insects: ecological and evolutionary contexts. *Adv. Insect Physiol.* **33**, 50–152. (doi:10.1016/S0065-2806(06)33002-0)
- Cossins, A. R. & Bowler, K. 1987 *Temperature biology of animals*. New York, NY: Chapman and Hall.
- Crnokrak, P. & Roff, D. A. 1999 Inbreeding depression in the wild. *Heredity* **83**, 260–270. (doi:10.1038/sj.hdy.6885530)
- Da Silva, A., Luikart, G., Yoccoz, N. G., Cohas, A. & Allainé, D. 2006 Genetic diversity–fitness correlation revealed by microsatellite analyses in European alpine marmots (*Marmota marmota*). *Conserv. Genet.* **7**, 371–382. (doi:10.1007/s10592-005-9048-y)

- Dahlgard, J. & Hoffmann, A. A. 2000 Stress resistance and environmental dependency of inbreeding depression in *Drosophila melanogaster*. *Conserv. Biol.* **14**, 1187–1192. (doi:10.1046/j.1523-1739.2000.99206.x)
- Falconer, D. S. & MacKay, T. F. C. 1996 *Introduction to quantitative genetics*, 4th edn. Harlow, UK: Longman.
- Fowler, K. & Whitlock, M. C. 1999 The distribution of phenotypic variance with inbreeding. *Evolution* **53**, 1143–1156. (doi:10.2307/2640818)
- Frankham, R. 2005 Genetics and extinction. *Biol. Conserv.* **126**, 131–140. (doi:10.1016/j.biocon.2005.05.002)
- Gibbs, A. G. 1999 Laboratory selection for the comparative physiologist. *J. Exp. Biol.* **202**, 2709–2718.
- Harshman, L. G. & Hoffmann, A. A. 2000 Laboratory selection experiments using *Drosophila*: what do they really tell us? *Trends Ecol. Evol.* **15**, 32–36. (doi:10.1016/S0169-5347(99)01756-5)
- Hedrick, P. W. & Kalinowski, S. T. 2000 Inbreeding depression in conservation biology. *Annu. Rev. Ecol. Syst.* **31**, 139–162. (doi:10.1146/annurev.ecolsys.31.1.139)
- Hoffmann, A. A. & Parsons, P. A. 1997 *Extreme environmental stress change and evolution*. Cambridge, UK: Cambridge University Press.
- Hoffmann, A. A., Sørensen, J. G. & Loeschcke, V. 2003 Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* **28**, 175–216. (doi:10.1016/S0306-4565(02)00057-8)
- Intergovernmental Panel on Climate Change (IPCC) 2007 *Impacts, adaptation and vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK: Cambridge University Press.
- Jamieson, I. G., Wallis, G. P. & Briskie, J. V. 2006 Inbreeding and endangered species management: is New Zealand out of step with the rest of the world. *Conserv. Biol.* **20**, 38–47. (doi:10.1111/j.1523-1739.2005.00282.x)
- Jensen, D., Overgaard, J. & Sørensen, J. G. 2007 The influence of developmental stage on cold shock resistance and ability to cold-harden in *Drosophila melanogaster*. *J. Insect Physiol.* **53**, 179–186. (doi:10.1016/j.jinsphys.2006.11.008)
- Keller, L. F. & Waller, D. M. 2002 Inbreeding effects in wild populations. *Trends Ecol. Evol.* **17**, 230–241. (doi:10.1016/S0169-5347(02)02489-8)
- Keller, L. F., Grant, P. R., Grant, B. R. & Petren, K. 2002 Environmental conditions affect the magnitude of inbreeding depression in survival of Darwin's finches. *Evolution* **56**, 1229–1239. (doi:10.1111/j.0014-3820.2002.tb01434.x)
- Kristensen, T. N., Dahlgard, J. & Loeschcke, V. 2003 Effects of inbreeding and environmental stress on fitness—using *Drosophila buzzatii* as a model organism. *Conserv. Genet.* **4**, 453–465. (doi:10.1023/A:1024763013798)
- Kristensen, T. N., Sørensen, A. C., Sørensen, D., Pedersen, K. S., Sørensen, J. G. & Loeschcke, V. 2005 A test of quantitative genetic theory using *Drosophila*—effects of inbreeding and rate of inbreeding on heritabilities and variance components. *J. Evol. Biol.* **18**, 763–770. (doi:10.1111/j.1420-9101.2005.00883.x)
- Kristensen, T. N., Sørensen, P., Pedersen, K. S., Kruhøffer, M. & Loeschcke, V. 2006 Inbreeding by environmental interactions affect gene expression in *Drosophila melanogaster*. *Genetics* **173**, 1329–1336. (doi:10.1534/genetics.105.054486)
- Kristensen, T. N., Loeschcke, V. & Hoffmann, A. A. 2007 Can artificially selected phenotypes influence a component of field fitness? Thermal selection and fly performance under thermal extremes. *Proc. R. Soc. B* **274**, 771–778. (doi:10.1098/rspb.2006.0247)
- Kristensen, T. N., Loeschcke, V. & Hoffmann, A. A. 2008a Linking inbreeding effects in captive populations to field performance—field releases with replicated *Drosophila melanogaster* lines under different temperatures. *Conserv. Biol.* **22**, 189–199. (doi:10.1111/j.1523-1739.2007.00816.x)
- Kristensen, T. N., Hoffmann, A. A., Overgaard, J., Sørensen, J. G., Hallas, R. & Loeschcke, V. 2008b Costs and benefits of cold acclimation in field released *Drosophila*. *Proc. Natl Acad. Sci. USA* **105**, 216–221. (doi:10.1073/pnas.0708074105)
- O'Grady, J. J., Brook, B. W., Reed, D. H., Ballou, J. D., Tonkyn, D. W. & Frankham, R. 2006 Realistic levels of inbreeding depression strongly affect extinction risk in wild populations. *Biol. Conserv.* **133**, 42–51. (doi:10.1016/j.biocon.2006.05.016)
- Pedersen, K. S., Kristensen, T. N. & Loeschcke, V. 2005 Effects of inbreeding and rate of inbreeding in *Drosophila melanogaster*—Hsp70 expression and fitness. *J. Evol. Biol.* **18**, 756–762. (doi:10.1111/j.1420-9101.2005.00884.x)
- Perry, A. L., Low, P. J., Ellis, J. R. & Reynolds, J. D. 2005 Climate change and distribution shifts in marine fishes. *Science* **308**, 1912–1915. (doi:10.1126/science.1111322)
- Pray, L. A., Schwartz, J. M., Goodnight, C. J. & Stevens, L. 1994 Environmental dependency of inbreeding depression: implications for conservation biology. *Conserv. Biol.* **8**, 562–568. (doi:10.1046/j.1523-1739.1994.08020562.x)
- Rako, L. & Hoffmann, A. A. 2006 Complexity of the cold acclimation response in *Drosophila melanogaster*. *J. Insect Physiol.* **52**, 94–104. (doi:10.1016/j.jinsphys.2005.09.007)
- Reed, D. H., Lowe, E. H., Briscoe, D. A. & Frankham, R. 2003 Fitness and adaptation in a novel environment: effect of inbreeding, prior environment, and lineage. *Evolution* **57**, 1822–1828. (doi:10.1111/j.0014-3820.2003.tb00589.x)
- Reed, D. H., Nicholas, A. C. & Stratton, G. E. 2007 Inbreeding levels and prey abundance interact to determine fecundity in natural populations of two species of wolf spider. *Conserv. Genet.* **8**, 1061–1071. (doi:10.1007/s10592-006-9260-4)
- Rutherford, S. L. & Lindquist, S. 1998 Hsp90 as a capacitor for morphological evolution. *Nature* **396**, 336–342. (doi:10.1038/24550)
- Spielman, D., Brook, B. W. & Frankham, R. 2004 Most species are not driven to extinction before genetic factors can impact them. *Proc. Natl Acad. Sci. USA* **101**, 15 261–15 264. (doi:10.1073/pnas.0403809101)
- Szulkin, M. & Sheldon, B. C. 2007 The environmental dependency of inbreeding depression in a wild bird population. *PLoS ONE* **2**, e1027. (doi:10.1371/journal.pone.0001027)
- Waller, D. M., Dole, J. & Bersch, A. 2008 Effects of stress and phenotypic variation on inbreeding depression in *Brassica rapa*. *Evolution* **62**, 917–931. (doi:10.1111/j.1558-5646.2008.00325.x)
- Walther, G.-R., Berger, S. & Sykes, M. T. 2005 An ecological 'footprint' of climate change. *Proc. R. Soc. B* **272**, 1427–1432. (doi:10.1098/rspb.2005.3119)
- Whitlock, M. C. & Fowler, K. 1999 The changes in genetic and environmental variance with inbreeding in *Drosophila melanogaster*. *Genetics* **152**, 345–353.
- World Conservation Monitoring Centre 1992 *Global biodiversity: status of the Earth's living resources*. London, UK: Chapman and Hall.