Equivalent inbreeding depression under laboratory and field conditions in a tree-hole-breeding mosquito

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Understanding the consequences of inbreeding has important implications for a wide variety of topics in population biology. However, most studies quantifying the effects of inbreeding are performed under artificial farm, greenhouse, laboratory or zoo conditions. Although several authors have argued that the deleterious effects of inbreeding (inbreeding depression) are likely to be more severe under natural field conditions than in artificial experimental environments, these arguments are usually speculative or based on indirect comparisons. We quantified the effects of inbreeding on fitness traits in a tree-hole-breeding mosquito (*Aedes geniculatus*) under near-optimal laboratory conditions and in three natural tree holes. Our index of fitness (R_0) was lower in the field than in the laboratory and declined due to inbreeding in both environments. However, we found no significant interactions between inbreeding depression and environmental conditions. In both the field and laboratory a 10% increase in the inbreeding coefficient (F) led to a 12–15% decline in fitness (R_0). These results suggest that inbreeding depression will not necessarily be more extreme under natural field conditions than in the laboratory.

Keywords: inbreeding depression; genotype $\times$ environment interaction; conservation genetics

1. INTRODUCTION

It has been known for centuries that mating between relatives generally produces offspring with reduced growth rates, increased mortality and lower fecundity (Darwin 1876). This reduction in the fitness of offspring from consanguineous mating is known as inbreeding depression and understanding the consequences of inbreeding depression is central to many areas of population biology. The evolution of mating systems (Charlesworth & Charlesworth 1987), dispersal strategies and social behaviour (Thornhill 1993), artificial breeding of agricultural stocks (Falconer & Mackay 1996) and the maintenance of rare and endangered species (Frankham 1995) can all be influenced by the effects of inbreeding depression.

Despite the fundamental importance of inbreeding depression in population biology, most studies examining the consequences of inbreeding are performed under artificial farm, greenhouse, laboratory or zoo conditions. Although several authors have argued that the effects of inbreeding are likely to be more severe under natural field conditions than in typical experimental environments, these arguments are either speculative (Ralls *et al.* 1988) or based on indirect comparisons (Keller 1998; Crnokrak & Roff 1999) rather than direct experimental evidence (but see Dudash 1990; Chen 1993; Jiménez *et al.* 1994). Several influential papers have stressed the importance of attempting to quantify the effects of inbreeding on composite indices of fitness for organisms raised under natural environmental conditions (Thornhill 1993; Frankham 1995).

We report on the effects of inbreeding in the mosquito *Aedes geniculatus* (Olivier), which lays its eggs and develops as a larva and pupa exclusively in the water-filled cavities

formed in deciduous hardwood trees (Kitching 1971; Yates 1979; Bradshaw & Holzapfel 1992). This discrete and naturally replicated habitat provides an excellent opportunity for performing experimental manipulations upon organisms reared in their natural environment. We maintained a control population (expected inbreeding coefficient $F=0$) and constructed replicate inbred lineages by single-pair, full-sib mating (44 lineages with expected $F=0.25$ and 37 lineages with expected $F=0.375$). We simultaneously raised experimental cohorts under near-optimal laboratory conditions and in larval cages in natural tree holes. We then measured the replacement rate (R_0), a composite index of fitness across the entire life cycle, in order to quantify the effects of inbreeding in the laboratory and in natural tree holes.

2. MATERIAL AND METHODS

(a) *Study organism and field site*

The range of *A. geniculatus* extends over 15° of latitude from northern Africa to Norway and from Britain eastwards to the Ukraine. Within the tree holes inhabited by pre-imago stages of *A. geniculatus*, decaying leaf litter and rotting wood provide the foundation of a food web consisting of fungi, bacteria, protists and aquatic insects (Srivastava & Lawton 1998). *A. geniculatus* is univoltine in Britain (Yates 1979; Bradshaw & Holzapfel 1992).

Two other species of mosquitoes, *Anopheles plumbeus* (Stephens) and *Culex torrentium* (Martini), also commonly inhabit tree holes in Britain, as do benthic detritivorous fauna including the scirtid beetle *Prionocyphon serriconis* (Müller), the chironomid midge *Metriocnemus martinii* (Thein) and the sryphid fly *Myiatropa florea* L. Previous studies have found no evidence of competitive interactions between the three common tree-hole-breeding mosquito species, indicating that these species effectively segregate their habitats in space and time (Bradshaw & Holzapfel 1992). The effects of the benthic fauna on the fitness of *A. geniculatus* are slight and facultative (Bradshaw & Holzapfel 1992). There are no known predators of the larval and pupal stages of *A. geniculatus* in Britain (Kitching 1971; Yates 1979; Bradshaw & Holzapfel 1992).

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All collections and field experiments were conducted in Epping Forest, London, UK (51°66' N, 0.00° 43' E). Epping Forest is an ancient woodland composed primarily of beech (*Fagus sylvatica* L.), with less abundant oak (*Quercus robur* L. and *Quercus petraea* Mattuschka) and hornbeam (*Carpinus betulus* L.), and with holly (*Ilex aquifolium* L.) and other species in the understorey.

(b) *Laboratory rearing*

All laboratory rearing and fitness assays were conducted under standardized conditions designed to provide a near-optimal environment for growth and development. The laboratory was maintained at a constant temperature of 21 ± 1 °C on a long-day photoperiod (18 L:6 D) (Simms & Munstermann 1983). Eggs were stored on moist paper towel in Petri dishes wrapped in plastic in order to maintain high (*ca.* 80%) humidity (Novack & Shroyer 1978). Developing larvae were reared at a density of *ca.* 20 larvae per 90 ml distilled water in 10 cm × 10 cm × 2 cm plastic Petri dishes. Larval food was prepared by blending 120 g of dried dog food (Beta-pet; Spillers Foods, Suffolk, UK) and 40 g of whole brine shrimp (Gammarus Foods, Herts, UK) in a kitchen food processor. The homogenate was mixed with 1-l distilled water and stored at 7 °C. Larvae were transferred to clean water every Monday, Wednesday and Friday and fed 1.5 ml of homogenized food. Pupae were removed, sexed and placed in sex-specific adult cages. Adults were kept in either a population cage consisting of an 18-l PVC cylinder (23 cm (height) × 50 cm (diameter)) with two 18 cm × 20 cm 1 mm mesh windows (control population) or 1-l plastic buckets with two 6 cm × 10 cm mesh windows. The cages were lined with moist paper towel at the bottom and provisioned with two organic raisins and 10% sucrose solution for adult nutrition (Clements 1963). Adult cages were wrapped in plastic in order to maintain a high (*ca.* 70%) relative humidity. In order to stimulate oviposition by fertilized females, a 20 ml black jar half full with tree-hole water and detritus, and lined with moist paper towel, was introduced to the cages eight days after the females had been artificially mated.

An outline of the breeding and experimental designs used in this study is presented in figure 1. Single-pair matings were performed throughout in order to maintain a control population and create inbred lineages. For each single-pair mating, an individual female was blood fed to repletion on a human host and artificially mated (McDaniel & Horsfall 1957) with a single male (no male was mated more than once). Initially, 300 larvae and pupae were collected from 35 tree holes in July 1998. Fifty randomly chosen females were then mated and placed in the 18-l population cage in order to establish the control population, and 45 randomly chosen females were mated and each placed in a separate 1-l cage in order to initiate inbred lineages (figure 1).

Eggs were transferred after one month in groups of *ca.* 30 to 3 ml glass vials submerged in 20 ml of 1g l^{-1} nutrient broth in order to stimulate larval hatch (Novack & Shroyer 1978; Simms & Munstermann 1983). Three hundred randomly chosen larvae from the control population were maintained in Petri dishes as described above and pupae removed to sex-specific cages. Fifty single-pair matings were performed in order to maintain the control population and a further 45 single-pair matings were performed in order to initiate a new set of inbred lineages (figure 1). Larvae from the eggs of single females (full-sibs) were reared in Petri dishes and pupae transferred to sex- and lineage-specific 1-l adult cages. Randomly chosen, full-sib pairs were then artificially mated and eggs collected from single females as

described above. These eggs were thus the product of one generation of full-sib mating (expected $F = 0.25$).

Eggs in the next generation were stimulated to hatch as described above. Control-line larvae were raised and 50 single-pair matings performed. Larvae from the eggs of females, which had been maintained individually for one generation, were raised and the resulting adults artificially mated in order to produce eggs with expected $F = 0.25$. Larvae from the eggs with expected $F = 0.25$ were raised to adulthood and a second generation of full-sib mating was performed in order to produce eggs with expected $F = 0.375$.

The control population in this experiment underwent three generations of laboratory, artificial mating during which the sex ratio was held constant (1:1) and all adults mated once (i.e. there was no variance in reproductive success). Assuming a Poisson distribution of family sizes, the effective population size (N_e) of the control population would be $N_e = 100$. The expected inbreeding coefficient of this population would thus be $F = 1 - (1 - 0.5 \times 100)^{-3}$ or $F = 0.015$ (Wright 1969). We refer to this population as the $F = 0$ treatment.

(c) *Field conditions*

Larvae were raised in the field in individual cohort cages submerged in natural tree holes. The cages were constructed from 20 cm lengths of cylindrical PVC tubing. Two cage sizes were used in order to increase the range of environmental variation in the field experiments. Large cages were 25 mm in diameter with two 10 cm × 4 cm windows cut out of the side. Small cages were 13 mm in diameter with 10 cm × 2 cm windows. Windows and the bottom of each cage were covered by 200 µm nylon mesh (BioDesign Inc., Carmel, NY, USA) attached with silicone sealant. Most mosquito larvae ingest food items less than 50 µm in size (Merritt *et al.* 1992). Four grams of leaf detritus were added to the bottom of each large cage. Therefore, larvae subjected to the large cage treatment could feed on micro-organisms in the water column of the cage and graze on micro-organisms growing on the leaf litter (Walker & Merritt 1991). No leaf detritus was added to the small cages. The top of each cage, which extended above the water line of the tree hole, was covered with 1 mm mesh held in place by a rubber band.

(d) *Comparison of caged versus uncaged mosquitoes in the field*

We assessed the effects of rearing larvae in cages in the field by comparing the mean mass of non-inbred female pupae ($F = 0$) reared in experimental cages with that of female pupae collected weekly from 12 unmanipulated tree holes in 1998 and 1999. A description of the method for collecting pupae from unmanipulated tree holes can be found in Bradshaw & Holzapfel (1992). We chose female pupal mass as an index of environmental conditions during growth and development because this trait is closely related to the size of the egg batch an individual produces, which is a primary component of fitness (R_0) in *A. geniculatus* (Hard *et al.* 1989). Furthermore, female pupal mass is known to be highly sensitive to environmental conditions during larval development both in *A. geniculatus* (Hard *et al.* 1989) and in other aedine mosquitoes (Clements 1963).

(e) *Measuring R_0 in the laboratory and field*

In order to quantify the effects of inbreeding on the fitness of *A. geniculatus* under laboratory conditions, eggs from each of the three inbreeding treatments (expected $F = 0.00, 0.25$ and 0.375)

Figure 1. Experimental protocol for establishing a control population and creating inbred lineages in order to measure inbreeding depression in the laboratory and in natural tree holes.

were simultaneously stimulated to hatch as described above. First-instar larvae were collected on the day of hatch and used for establishing experimental cohorts of 15 larvae each. Fifteen cohorts were established for the control population. Eight first-instar larvae from each of the 22 lineages were pooled for the $F = 0.25$ treatment and ten cohorts, each of 15 randomly chosen individuals, were established. Five first-instar larvae from each of 18 lineages were pooled for the $F = 0.375$ treatment and six cohorts, each of 15 randomly chosen individuals, were established.

Not all viable eggs of *A. geniculatus* responded to the first hatching stimulus (Simms & Munstermann 1983). Therefore, it was necessary to restimulate eggs in order to assess fertility (percentage hatch). Eggs were stimulated to hatch at biweekly intervals a total of seven times, and fertility (percentage hatch) was calculated as the number of first-instar larvae to hatch from a batch of eggs after seven stimulations divided by the initial egg batch size.

Larvae were raised as described above (see § 2(b)). Pupae were collected every Monday, Wednesday and Friday, gently placed on a paper towel in order to remove excess water and then weighed to the nearest 0.01 mg. Larval survivorship was calculated as the number of pupae to develop from a cohort divided by the initial cohort size. Female pupae were placed in individual adult cages after weighing. Adult females were blood fed and five to eight days later female ovaries were dissected and the number of mature (stage IV) egg follicles counted. Egg batch size was then defined as the number of mature follicles per female (Packer & Corbet 1989) and weight-specific fecundity was calculated as egg batch size divided by female pupal weight.

We combined individual traits in order to calculate a composite index of fitness, i.e. the replacement rate (R_0). The replacement rate (R_0) estimates the number of offspring produced in the next generation per individual in each cohort and provides the most appropriate composite index of fitness available for univoltine populations (Charlesworth 1980). R_0 was calculated

Table 1. *Analyses of variance on fitness traits in A. geniculatus*

(Environment (laboratory or field) and inbreeding ($F = 0, 0.25$ and 0.375) were analysed as fixed effects. Fertility was measured under laboratory conditions only (see the text and figure 1). Statistical inference based on type III sum of squares.)

fitness trait	source of variation	d.f.	sum of squares	F	p -value
fertility (% egg hatch)	inbreeding	2	783 009.00	22.68	< 0.0001
	error	148	2 554 689.00	—	—
larval survivorship	environment	1	1551.93	12.65	0.0007
	inbreeding	2	5530.83	22.54	< 0.0001
	environment $\times$ inbreeding	2	644.34	2.63	0.0795
	error	70	8586.90	—	—
female pupal weight	environment	1	2.83	48.66	< 0.0001
	inbreeding	2	0.18	1.57	0.2101
	environment $\times$ inbreeding	2	0.29	2.49	0.0849
	error	300	17.43	—	—
weight-specific fecundity	environment	1	1.89	0.34	0.5626
	inbreeding	2	4.33	0.39	0.6798
	environment $\times$ inbreeding	2	2.47	0.22	0.8025
	error	132	738.99	—	—
replacement rate (R_o)	environment	1	4.87	11.73	0.0010
	inbreeding	2	19.49	23.50	< 0.0001
	environment $\times$ inbreeding	2	1.38	1.66	0.1972
	error	70	29.03	—	—

for each cohort as female pupal weight $\times$ weight-specific fecundity $\times$ fertility summed over all female pupae to develop from a cohort and divided by the initial cohort size.

The field experiment was initiated on 7 April 1999, one day after the laboratory experiment was established and approximately the mean hatch date of the natural population in Epping Forest. Eggs were simultaneously stimulated to hatch and 17 randomly chosen, first-instar larvae were selected for establishing each of 18 replicate cohorts for the control population. Sixteen larvae from each of 22 lineages with expected $F = 0.25$ were pooled, and 17 individuals were randomly selected for establishing each of 18 replicate cohorts. Seventeen larvae from each of 19 lineages with expected $F = 0.375$ were pooled, and again 17 individuals were randomly selected for establishing each of 18 replicate cohorts. As in the laboratory experiment, eggs were restimulated a total of seven times in order to assess fertility. First-instar larvae were transferred to the field on the day of hatch. The journey took *ca.* 1 h with the larvae in 100 ml vials in a cooler maintained at 12 °C.

Three replicate cohorts for each of the two cage size treatments and each of the three levels of inbreeding were established in each of three beech tree holes (one pan hole and two rot holes as described by Kitching (1971)) (see figure 1). Cages were checked in order to assess larval development once after three weeks and after five weeks and thereafter once a week until fourth-instar larvae were apparent. Cages were then checked twice weekly. Pupae were collected, transferred back to the laboratory in 100 ml vials in a cooler at *ca.* 12 °C, sexed and weighed. Female pupae were then transferred to individual adult cages and egg batch size was determined as described above for the laboratory experiment. Fitness components and R_o were calculated for each cohort established in the field as described for the laboratory experiment.

(f) *Preliminary analyses and statistical methods*

There were no significant differences in weight-specific fecundity between the inbreeding treatments for females reared as larvae in either the field or laboratory (table 1 and figure 4c).

We therefore pooled across inbreeding treatments (but not environments) and used the regression of egg batch size on pupal weight (PW) in order to estimate the egg batch size of all female pupae reared under laboratory (egg batch size = $30.29 - 2.8 \times PW + 1.52 \times PW^2$, $R^2 = 0.56$ and $p < 0.001$) and field (egg batch size = $32.73 + 1.17 \times PW + 0.81 \times PW^2$, $R^2 = 0.62$ and $p < 0.001$) conditions.

In one of the six field-environment treatments (i.e. small cages in one of three tree holes) the replacement rate (R_o) was zero in seven out of nine cages (two $F = 0$, two $F = 0.25$ and three $F = 0.375$). These data were uninformative and excluded from further analysis.

ANOVA was performed in S-Plus v. 4.5 (1997) after transformation of the data in order to approximate a normal distribution and stabilize variances between treatments. Fertility and larval survivorship data were square root-arcsine transformed. Female pupal weight data were log transformed and no transformation was necessary for the weight-specific fecundity data. Because several R_o -values were zero, the replacement rate (R_o) data were subjected to the transformation $\ln(R_o + 1)$.

3. RESULTS

(a) *Experimental and census data from the field*

The mean mass of control population ($F = 0$), female pupae in the remaining five field-environment treatments and the distribution of female pupal mass from 12 tree holes over two years are presented in figure 2. The experimental treatments used in this study produced female pupae whose weights spanned the natural distribution of female pupae developing in Epping Forest. Therefore, our field experiments imposed a natural level of environmental variation.

(b) *Fitness in the laboratory and field*

With the exception of female pupal mass, all data were approximately normally distributed and the variances were

Figure 2. The distribution of female pupal weights from 12 unmanipulated tree holes censused weekly in 1998 and 1999 (filled squares). Vertical arrows indicate the mean female pupal weight of non-inbred cohorts (expected $F = 0$) in five experimental field environments (i.e. specific combinations of cage size and tree hole).

Figure 3. Fertility (arcsine-square-root transformed ± 2 s.e.s) in three inbreeding treatments under laboratory conditions. The results of the ANOVA given in the upper right-hand corner indicate the effects of inbreeding (Inb). *** $p < 0.001$.

Figure 4. The effects of inbreeding and environment (filled circles, laboratory and open circles, field) on (a) larval survivorship (arcsine-square-root transformed), (b) female pupal weight ($\log_e$ transformed), (c) weight-specific fecundity, and (d) replacement rate (R_0 subjected to the transformation $\ln(R_0 + 1)$). All points represent means ± 2 s.e.s. The results of the ANOVA testing for the effects of environment (Env), inbreeding (Inb) and environment $\times$ inbreeding interaction ($E \times I$) are given in the upper right corner. $^{ns}p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

homogeneous between treatments (Cochran's test $p > 0.05$). The variances in female pupal mass were significantly heterogeneous between treatments (Cochran's test $p < 0.01$) and no transformation was sufficient for stabilizing these variances. However, the effect of heterogeneous variances is

to increase the probability of type I errors. Because the only significant effect in the analysis of the female pupal weight data was significant at the $p < 0.001$ level (see table 1), the conclusions regarding significance in this analysis remain robust (Underwood 1981).

The results of the ANOVA examining the effects of environment, inbreeding and environment $\times$ inbreeding interaction are summarized in table 1. Fertility (which was only assessed in the laboratory) (see figure 1) displayed substantial inbreeding depression (table 1 and figure 3). Larval survivorship was lower in tree holes than in the laboratory and suffered substantial and similar levels of inbreeding depression in both environments (table 1 and figure 4a). Female pupae from tree holes weighed less than female pupae from the laboratory, but this trait was not affected by inbreeding depression or an environment $\times$ inbreeding interaction (table 1 and figure 4b). Weight-specific fecundity was not affected by environment, inbreeding or environment $\times$ inbreeding interaction (table 1 and figure 4c). The replacement rates (R_0) of cohorts raised in tree holes were lower than those of cohorts raised in the laboratory. R_0 exhibited high levels of inbreeding depression (an *ca.* 12–15% decrease per 10% increase in F), but was not affected by an environment $\times$ inbreeding interaction (table 1 and figure 4d).

In summary, the lower performance (larval survivorship, female pupal weight and R_0) in the natural tree holes than in the laboratory clearly indicated that the natural environment was more stressful than the laboratory. Furthermore, there was substantial inbreeding depression in *A. geniculatus* in both the laboratory and natural environments. However, we found no significant inbreeding $\times$ environment interactions.

4. DISCUSSION

To the authors' knowledge, this is the first study to quantify the effects of inbreeding in a mosquito in detail. The population of *A. geniculatus* examined in this study suffered high levels of inbreeding depression in both the laboratory and under natural field conditions (figures 3 and 4a–d). The replacement rate (R_0) declined by 12–15% per 10% increase in the inbreeding coefficient in both natural tree holes and the laboratory (figure 4d).

Many studies of inbreeding depression in the wild have been equivocal (see table 1 in Keller (1998)). In some cases, this may have been due to the difficulties in performing controlled experiments in a natural environment. These equivocal results have led some authors to argue that there is no good evidence for inbreeding depression under natural field conditions (Shields 1993). However, we found substantial inbreeding depression in *A. geniculatus* reared in their natural tree-hole environment (figure 4a–d).

Several authors have argued that inbreeding depression should be more severe in natural environments than under typical experimental conditions. However, there is very little direct experimental evidence addressing this issue. These arguments are intuitively appealing based on the generalization that inbreeding depression is often more severe under adverse conditions than in benign environments (Frankham 1995). However, this result is not very robust. Stressful environments do sometimes increase the magnitude of inbreeding depression (Schmitt & Ehrhardt 1990; Wolfe 1993; Miller 1994; Bijlsma *et al.* 1999), but often they do not (Waller 1984; Mitchell-Olds

& Waller 1985; Miller 1994; Dahlgaard *et al.* 1995; Hauser & Loeschke 1996; Dahlgaard & Loeschke 1997).

A study in plants by Dudash (1990) found increased inbreeding depression for overall fitness in a field environment relative to greenhouse conditions. Two previous studies directly compared inbreeding depression in animals under laboratory and natural field conditions. Both Jiménez *et al.* (1994) (for white-footed mice) and Chen (1993) (for a land snail) found evidence of inbreeding depression for survivorship in a natural environment but not under laboratory conditions. However, such individual components of fitness can often be poor indicators of overall fitness due to complex trade-offs between life-history traits (Roff 1992), as has been documented in *A. geniculatus* (Hard *et al.* 1989). Many authors have emphasized the importance of measuring composite fitness indices across the whole life cycle when assessing the effects of inbreeding (see Thornhill 1993). Our results from examining the replacement rate (R_0) rather than a single fitness component (e.g. survivorship) indicated that, in *A. geniculatus*, the effects of inbreeding depression were similar in both magnitude and timing across the life cycle under near-optimal laboratory conditions and in natural tree holes (figure 4a–d). These results indicate that inbreeding depression will not necessarily be more extreme under natural field conditions than in benign laboratory environments.

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