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# Two classes of deleterious recessive alleles in a natural population of zebrafish, *Danio rerio*

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Natural populations carry deleterious recessive alleles which cause inbreeding depression. We compared mortality and growth of inbred and outbred zebrafish, *Danio rerio*, between 6 and 48 days of age. Grandparents of the studied fish were caught in the wild. Inbred fish were generated by brother–sister mating. Mortality was 9% in outbred fish, and 42% in inbred fish, which implies at least 3.6 lethal equivalents of deleterious recessive alleles per zygote. There was no significant inbreeding depression in the growth, perhaps because the surviving inbred fish lived under less crowded conditions. In contrast to alleles that cause embryonic and early larval mortality in the same population, alleles responsible for late larval and early juvenile mortality did not result in any gross morphological abnormalities. Thus, deleterious recessive alleles that segregate in a wild zebrafish population belong to two sharply distinct classes: early-acting, morphologically overt, unconditional lethals; and later-acting, morphologically cryptic, and presumably milder alleles.

**Keywords:** inbreeding depression; lethal equivalent; variance; mortality; growth rate; zebrafish

## 1. INTRODUCTION

Inbreeding depression is ubiquitous in plants and animals (Charlesworth & Charlesworth 1987). To a large extent, inbreeding depression is caused by homozygosity of recessive deleterious alleles in the offspring of consanguineous matings (Morton *et al.*1956; Charlesworth & Charlesworth 1999; Roff 2002). Whereas an average genotype from a natural population can carry many deleterious alleles (Sunyaev *et al.* 2001), most of them are rare in all but the smallest populations, and thus become homozygous only in inbred individuals (Crow 1979).

The properties of deleterious alleles are quite varied. A heterozygote can be unaffected (complete recessivity) or, more often, have a somehow reduced fitness. The fitness of a homozygote can be zero (completely penetrant lethals) or only mildly reduced. A deleterious allele can begin to affect fitness at any stage of individual development. Some alleles cause drastically abnormal phenotypes, whereas many others are morphologically cryptic (see Timofeeff-Ressovsky 1935; Mukai *et al.*1972; Crow & Simmons 1983).

Recessive mutations that do not cause clear-cut phenotypes can be assayed through inbreeding depression. Data on fitnesses of individuals with different coefficients of inbreeding make it possible to estimate, without resolving the mutational damage into individual alleles, the genomic number of lethal equivalents (LEs). An LE is defined as a set of alleles that, if dispersed in separate individuals and made homozygous, would cumulatively cause one genetic death (Morton *et al.* 1956). More elaborate analyses can reveal the genomic number of deleterious alleles (Deng & Lynch 1996; Charlesworth & Hughes 2000; Rudan *et al.* 2003).

Measurements of inbreeding depression have produced many estimates of the number of LEs. In humans, limited data indicate only a moderate (less than 1) per zygote number of LEs of alleles that cause early prenatal mortality (see Shull & Neel 1972; Hussain 1998*a*). This is probably an underestimate, because preclinical pregnancy losses often remain unnoticed (Macklon *et al.* 2002). A human zygote carries 1.5–5 LEs of alleles causing late prenatal, neonatal, infant and juvenile deaths (Morton *et al.* 1956; Chakraborty & Chakravarti 1977; Revazov 1983; Revazov *et al.* 1984; Khoury *et al.* 1987; Bittles & Neel 1994; Lee *et al.* 1996; Wahab & Ahmad 1996; Hussain 1998*b*; Yaqoob *et al.* 1998; Jorde 2001). The number of LEs detected may be lower in more recent studies because of the impact of improved living conditions and healthcare on the phenotypic manifestations of incompletely penetrant alleles (discussed in Morton *et al.* 1956; Revazov 1983; Bittles & Neel 1994; Meagher *et al.* 2000; Kalinowski & Hedrick 2001). Recessive alleles that reduce fecundity of adults are also common (Ober *et al.* 1999).

Data on other vertebrates produce rather diverse estimates of the number of LEs (see table 6 in Keller *et al.* 2002), perhaps reflecting the varying severity of conditions under which fitness was assayed (see Jimenez *et al.* 1994; Keller & Waller 2002; Joron & Brakefield 2003), as well as diverse population histories (Duarte *et al.* 2003). Among mammals, there are 12.6 LEs per zygote of alleles reducing three week adult survival in white-footed mice, *Peromyscus leucopus* (Jimenez *et al.* 1994). In birds, song sparrows (*Melospiza melodia*) carry at least three LEs of alleles affecting viability from conception until independence from parental care, and 2.5 LEs of alleles affecting later fitness (Keller 1998). Data on three species of Darwin finches (*Geospiza*) inidicate, on average, about six LEs of alleles affecting juvenile survival and even more severe inbreeding

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depression later in life (Keller *et al.* 2002). In total, there are *ca*. 15 LEs, affecting all components of fitness through all stages of life, in the collared flycatcher *Ficedula albicollis* (Kruuk *et al.* 2002). Data on invertebrates (see, for example, Coutellec-Vreto *et al.* 1998; Launey & Hedgecock 2001; Haag *et al.* 2002; Kristensen *et al.* 2003; Joron & Brakefield 2003) and plants (see, for example, Charlesworth *et al*. 1994; Remington & O'Malley 2000) are generally consistent with estimates obtained for vertebrates.

In the wild populations of two species of fishes, bluefin killifish (*Lucania goodei*) and zebrafish (*Danio rerio*), an individual carries on average *ca*. 1.5–2.0 morphologically overt, early-acting, completely penetrant recessive lethals (McCune *et al.* 2002), which is in line with data on *Xenopus laevis* (Krotoski *et al.* 1985) and several species of *Drosophila* (Lewontin 1974). Of the 55 early-acting recessive lethals detected in these two fishes, *L. goodei* and *D. rerio*, none were morphologically cryptic. Here, using the same sibships of *D. rerio* where early-acting lethals were studied earlier (McCune *et al.* 2002), we investigate inbreeding depression in mortality and growth rate during late larval and early juvenile development.

## 2. MATERIAL AND METHODS

### (a) *Experimental animals*

Wild-caught *D. rerio* (generation P (Pelevin 1998)) were obtained through Asian Exports of Calcutta, India, in 2000, and produced 15 $F_1$ families in captivity. Ethanol-preserved voucher specimens of wild-caught individuals have been deposited in the Cornell University Museum of Vertebrates (CU 89303). No P fish was used to produce more than a single $F_1$ family. We compared viability and growth of inbred $F_2$ individuals, produced by brother–sister matings, and of outbred controls (figure 1). $F_1$ sibships used in the current study are the same as in a study of early-acting lethals, and are described in McCune *et al.* (2002).

Eggs were obtained from 10 brother–sister and 10 unrelated pairs of $F_1$ fish. Inbred $F_2$ fish were obtained from additional matings of pairs of $F_1$ sibs (obtained from family numbers 3, 4, 5, 6, 8, 9, 10, 13, 14 and 15) used in the previous study. To produce outbred $F_2$, males and females from different $F_1$ families (1, 4, 8, 9, 10, 11, 13, 14 and 15) were crossed. No $F_1$ individual participated in more than one pair, although in several cases, different $F_1$ individuals from the same family were used to make outbred crosses in different family combinations. Representation of families in both the inbred and outbred $F_1$ pairs created for this study was determined by availability of fish and which fish gave eggs.

For most brother–sister pairs, it was known from previous work (McCune *et al.* 2002) that the male and female of a couple did not carry the same early-acting recessive lethal and thus no such lethals would be expressed in $F_2$. However, three such pairs produce offspring of which 25% would express a recessive lethal. For the clutches produced by these three pairs, individuals homozygous for the lethal were removed before the larvae began feeding and were replaced with extra individuals, not expressing the lethal, from the same clutch.

$F_2$ clutches were split and reared under a high or low food regimen (figure 1). Growth of individuals was monitored weekly, and mortality was monitored daily for both treatments until day 30, when the fish were transferred from rearing boxes to a zebrafish rack system, and then on days 34, 41 and 48.

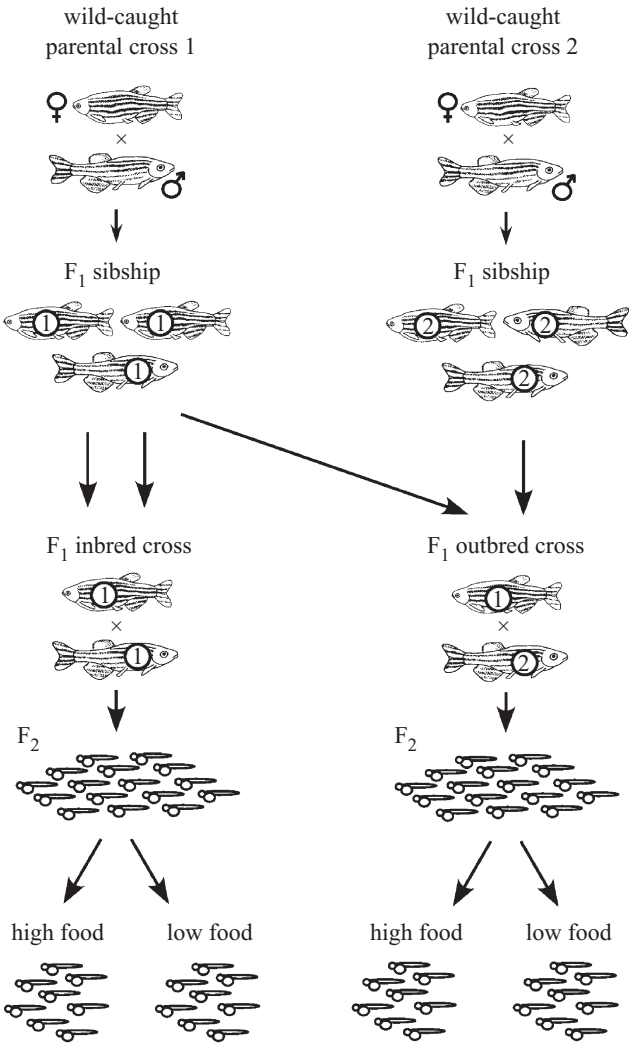


Figure 1. Experimental design. $F_1$ families were created from wild-caught fish. Ten clutches of $F_2$ inbred fish were produced through $F_1$ sib matings. Ten experimental outbred clutches were produced from $F_2$ fish, derived from outcrosses between different $F_1$ families. Forty fry were reared from each $F_2$ clutch, whether inbred or outbred, with 20 fish subjected to either a high food or low food treatment. All surviving individuals from the initial set of 800 were imaged and measured weekly from day 6 to day 48.

### (b) *Rearing protocol*

Clutches of eggs were divided into at least two groups of 20 and each group kept in 100 × 15 mm Petri dishes containing embryo medium (Westerfield 1995). One Petri dish was arbitrarily assigned to high food treatment and one dish was assigned to low food treatment. Extra eggs from a clutch were maintained in groups of 20 and used to replace embryos homozygous for recessive lethals before fry started feeding ($F_1$ pairs 8–5, 10–4 and 13–7). We also replaced a single dead individual in two clutches (11–7 and 10–4) before fry started feeding. Given possible effects of fish density on growth, we originally planned to replace dead fish during the experiment with extra fish reared under the same conditions to maintain constant fish density in treatment groups, but it proved to be impossible to control both food treatment and density in extra groups of fry to serve as replacements. Thus, we only replaced dead individuals after fry started feeding in the first two clutches produced (inbred 15 × 15 (clutch 1) and outbred

$15 \times 4$). Given the likely possibility that food density (and thus amount of food locally available to an individual) rather than simply the absolute amount of food was an important factor determining food availability to individuals, the volume of water was standardized throughout the experiment. On day 6, the fry in each Petri dish were transferred to a 2 l plastic box containing 0.5 l water, with the volume of water being increased to 1 l on day 9. On a daily basis, leftover food (from both treatments) was removed from the bottoms of boxes by suction and all but 200 ml of water in each box was replaced with clean water. Fry were transferred to clean boxes on days 13 and 20. On day 30, fry were moved to a 2.7 l plastic box on a zebrafish rack system made by Aquatic Habitats, Apopka, FL, USA. Once on the rack, system water was filtered automatically. Excess food continued to be removed daily from containers of fish receiving both treatments. All fish of all developmental stages were reared and maintained at 28.5 °C and kept on a 13 L : 11 D cycle.

### (c) *Food treatments*

Feeding protocols for high and low food treatments were worked out by trial and error on offspring from outcrossed $F_1$ parents. The high food treatment was selected to maximize growth during the first 14 days. With dried food, feeding larger amounts seemed to depress growth, perhaps because of increased fouling of the water. Low food treatment was chosen to produce an apparent difference in growth from the high food treatment, without resulting in mortality of outbred fry. Inbred fish were not tested on experimental diets before the experiment.

Experimental fish were fed twice daily, starting on day 4, just before the time that the yolk sac of most individuals is depleted. From days 4 to 10, we fed all experimental fish a nutritionally complete dry food, ZM fry feed (Zebrafish Management Ltd., Hampshire, UK) available in graded sizes appropriate for different sized larvae. On days 4 and 5, equal quantities of ZM-000, amounting to the volume covering 4 $mm^2$ of the corner of a spatula ($< 0.01$ g), was given to both high and low treatments. Once the fry were transferred to boxes, fry were given weighed measures of food, with the amount of the high food treatment being approximately twice that on low food treatments. Starting on day 9, fry were fed the larger ZM-100. Beginning on day 11, fry were fed increasingly large amounts of brine shrimp. The amount of ZM food given was gradually reduced starting on day 15, and none was given after day 17. Starting on day 30, fish were given flake food for one daily feeding and brine shrimp for the other feeding. The exact feeding and care protocols are detailed in electronic Appendix A.

### (d) *Measurement and morphology*

Fish were measured weekly starting on day 6 until day 48. Identities of individual fish were not tracked over sampling dates. To obtain measurements, the fish in each Petri dish or box were anaesthetized in MS-222 (tricaine) as described in Westerfield (1995). Images of individual fish were recorded by using an Optronics Magnafire SP digital camera attached to a Wild M-8 stereomicroscope using transmitted light in addition to oblique lighting from four fibreoptic lights or a ring light. The length of each individual on a particular day was taken using the software, Image Pro Plus, v. 4.5. In dorsal view, the length of the fish was taken as the distance between the dorsal tip of the snout and the tip of the caudal fin. In lateral view, this length corresponded to the length between the dorsal-most tip of the snout and the most posterior extent of the third most dorsal caudal fin ray. Anaesthetized fish up to 20 days were photographed in dorsal view. Fish older than 34 days were photographed in lateral view because by that time, the shape of the fish caused the fish to lie on their sides when anaesthetized. On day 27, fish were mostly imaged in lateral view, although some were sufficiently less developed that they were still imaged in dorsal view.

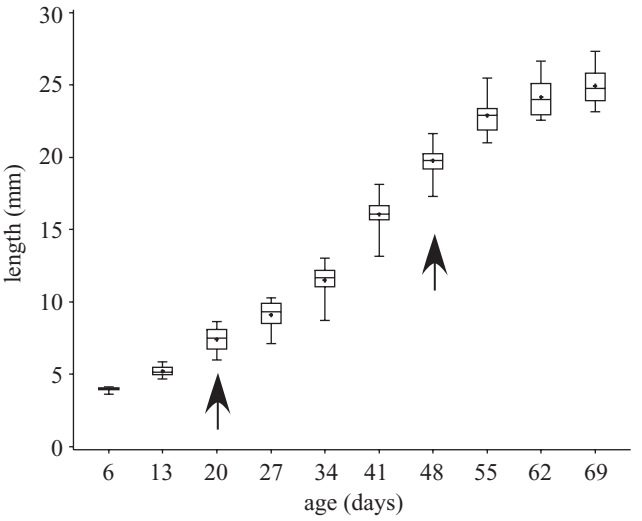


Figure 2. Representative growth curve for an outbred clutch (sibships $11 \times 10$, high food treatment) showing sampling points. Fish were measured weekly starting on day 6, just after fish began to feed. Identities of individual fish were not tracked between sampling dates. Statistical analyses were performed on day 20 to assess early growth effects and day 48 to assess later growth effects. Day 27 was avoided for statistical analyses because around that time the shape of the fish changes so that anaesthetized fish begin to lie on their sides rather than remaining vertical, resulting in possible spurious variation in measurement. Arrows show sampling days subjected to statistical analyses.

At the completion of the experiment, stored images were again screened for abnormal morphology. Because 70% of inbred mortality occurred between days 6 and 20, all *ca*. 400 images of 6 day old fish and all images of *ca*. 350 surviving 13-day-old fish were reviewed carefully for abnormal morphology. At day 6, the presence of the gas bladder in every individual was verified because most recessive lethals previously discovered (22 out of 26) have involved loss or non-inflation of the gas bladder (McCune & Carlson 2004). Images of 13-day-old fish were screened generally for morphology and particularly for mutant phenotypes known to occur in other members of its family.

### (e) *Data analysis*

We used the Mixed procedure of SAS (2001) to examine whether food abundance or inbreeding affects size and mortality. As a baseline, fixed effects on size were tested on day 6, just as the fish were beginning to feed. Fixed effects on growth were then tested on day 20, when fish had been feeding for two weeks, to assess effects on early growth, and also at day 48, well into the rapid growth phase and before the slope of the growth curve starts to decline (figure 2). Inbreeding (inbred versus outbred) and food abundance (high versus low) were treated as fixed effects in both the size and mortality models. Family was treated as a random effect.

Viability data involves binomial variation, as well as the usual sources of variation. Binomial variance scales with the mean as well as sample size, making analyses assuming normally distributed errors inappropriate. In addition, our design involves the use of both fixed effects (level of inbreeding and food level) and random sampling of inbred and outbred families. Proper analyses take both of these complications into account. Consequently, analysis of the viability data was accomplished using a generalized linear mixed model (Littell *et al*. 1996, ch. 11). This was accomplished using the GLIMMIX macro in SAS, which implements the approach of Wolfinger & O'Connell (1993). This macro iteratively runs the MIXED procedure in SAS on data transformed in the link function appropriate to the data, in our case the logit function. Unfortunately, the variance components estimated by GLIMMIX are on the logit scale, which have no simple relationship to variances on the probability scale that interests us. To obtain among family variances on the probability scale appropriate for estimating the minimum number of factors we used restricted maximum likelihood in the VARCOMP procedure of SAS.

### (f) *Number of lethal equivalents*

The rate of decline in fitness with inbreeding can be used to estimate the total number of LEs per individual (Morton *et al.* 1956). The number of LEs is the total number of genetic deaths that would result if all of the alleles in an individual were made homozygous. Some of these deaths are expressed even in an outbred population, and are difficult to measure. Most LEs are expressed only upon inbreeding and can be estimated as twice the rate of increase in mortality with inbreeding. When comparing non-inbred individuals with individuals inbred to the same degree, $F$, the rate of increase in mortality with inbreeding is

$$B = -\frac{1}{F}\ln\left(\frac{W_{\mathrm{I}}}{W_{\mathrm{O}}}\right),$$

where $W_{\mathrm{O}}$ is the fitness of outbred individuals and $W_{\mathrm{I}}$ the fitness of inbred individuals. After full-sib mating, $F = 0.25$. The number of LEs per genome is $2B$.

### (g) *Minimal number of factors responsible for inbreeding depression*

We estimated $n$, the minimum number of partly deleterious factors responsible for late larval–early juvenile mortality from the variance in viability using the approach of Rudan *et al.* (2003), a generalization of previous work (Mukai *et al.* 1974; Charlesworth & Hughes 2000). Rudan *et al*. show that as long as any variance component contributed by the $i$th locus ($V_{\mathrm{G}i}$) is greater than or equal to the inbreeding depression ($W_i$) contributed by that locus, then

$$n \geqslant \frac{(\sum_i \delta W_i)^2}{\sum_i V_{\mathrm{G}i}} = \frac{(\delta W)^2}{V_{\mathrm{G}}}.$$

The dominance variance at the $i$th locus is equal to $W_i$ (Charlesworth & Hughes 2000). From the data available, we can estimate the total genetic variance among outbred families, which is the sum of additive and dominance genetic variances, and thus fulfils the necessary conditions. We estimated the variance among outbred $F_2$ families using the VARCOMP procedure in SAS. To do this we took advantage of the fact that the food treatment had no effect on mortality, so that these split broods could be used to estimate the effect of a common rearing environment.

Table 1. Results of the generalized linear mixed model testing the fixed effects on survival.

| effect | num. d.f. | denom. d.f. | $F$-value | $p > F$ |
|---|---|---|---|---|
| inbreeding | 1 | 19 | 20.79 | 0.0002 |
| food | 1 | 17 | 1.74 | 0.20 |
| inbreeding by food | 1 | 17 | 0.67 | 0.42 |

### (h) *Timing of death as a result of partially deleterious factors*

We used a Mann–Whitney $U$-test to assess whether timing of deaths caused by morphologically drastic recessive lethals differed from timing of deaths as a result of partially deleterious, morphologically cryptic alleles.

## 3. RESULTS

### (a) *Morphology*

No abnormal morphology was noted in any individual fish during weekly imaging, although each individual fish was anaesthetized, positioned under a dissecting scope and imaged seven times over the course of its development. Subsequent review of nearly 700 images taken on days 6 and 13, revealed no morphological abnormalities visible in dorsal view. In particular, we verified that all individuals had inflated gas bladders, and no individuals expressed phenotypes known to occur in other family members. Aberrant morphologies only visible in lateral view could have been missed in the course of screening of dorsal view images, although such morphologies, unless subtle, would probably have been detected during the earlier process of recording images.

### (b) *Mortality*

Each of the families in the experiment was reared in two tanks differing in the amount of food furnished to the developing fish. A random sample of inbred and outbred families was studied, so the fixed effects of inbreeding and food levels must be tested against the background of between-family variation.

The first step in our analysis was to compare models that allowed the level of family variance to differ with inbreeding. Parameter estimates of the variance of inbred and outbred families on the logit scale were virtually identical, and the model with different variances had a higher Akaike information criterion (115.8 versus 113.8). Hence, we tested the fixed effects in a model with one family variance parameter.

The results of tests of the fixed effects are shown in table 1. Food had no significant effect on survival, although the least squares means showed that survival was slightly higher in the high food treatment (81% versus 76%). The effect of inbreeding was highly significant, and the least squares means showed a large difference in survival between outbred and inbred families. Mortality of outbred families was only 9% versus 42% for the inbred families (figure 3). Inbreeding depression, $W$, was thus 33% on the probability scale, or a 36% decrease in survival with sib-mating from random mating. There was also no evidence that families responded differently to the food treatment. The error variance was 1.3 times as large as the expected binomial variance, suggesting that there were random tank effects.

Table 2. Results of the mixed model analysis of fixed effects on size at days 20 and 48.

| day tested | effect | num. d.f. | denom. d.f. | *F*-value | $p > F$ |
|---|---|---|---|---|---|
| day 20 | inbreeding | 1 | 18 | 5.98 | 0.025 |
| | food | 1 | 644 | 10.47 | 0.0013 |
| | inbreeding by food | 1 | 644 | 1.21 | 0.27 |
| day 48 | inbreeding | 1 | 18 | 0.23 | 0.64 |
| | food | 1 | 571 | 15.83 | $< 0.0001$ |
| | inbreeding by food | 1 | 571 | 0.06 | 0.81 |

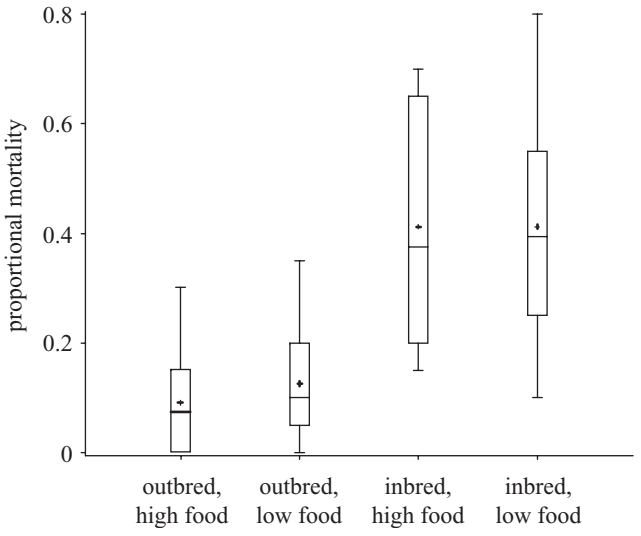


Figure 3. Box and whisker plot for mortality data at day 48. Boxes show 50% of the data, whiskers give the range, a plus symbol denotes the mean, and the horizontal line within the box, the median. Note that inbred clutches sustained *ca*. 42% mortality, whereas outbred clutches suffered *ca*. 9% mortality.

From these data, we estimate that the number of LEs per zygote is 3.6. If there is genetic variance within the outbred population for survival, this is a slight underestimate of the true impact of genetic variation on survival.

### (c) *Minimal number of factors responsible for inbreeding depression in viability*

As described above, the minimal number of factors contributing to inbreeding depression can be estimated. Although we cannot directly estimate the dominance variance in our design, we can estimate the total genetic variance among families. The lack of significant food effects on survival allowed us to estimate the variance in survival among families after tank effects are removed. On the original viability scale, the variance among outbred families was $V_G = 0.0064$. Generalized mixed model analysis showed that the variance among outbred families was not quite significant ($p = 0.088$), suggesting that this estimate is not very precise. Our estimate of the minimal number of partly recessive factors affecting viability in the population is

$$n \geqslant \frac{(0.33)^2}{0.0064} = 17.0.$$

Because the total genetic variance is larger than the dominance variance, this already minimal estimate is further biased downwards.

### (d) *Growth*

At the beginning of the experiment (day 6), there was significant variation among families in size ($0.015 \pm 0.005$), but no significant size differences among treatments (data not shown). Consequently, we assumed that the sizes of fish primarily reflected their growth rate over the course of the experiment. Tests of the fixed effects on size on two dates, reflecting two phases of growth (figure 2), is shown in table 2. On both dates, there was highly significant variance in size among families. However, despite the non-independence of testing the same fish on these two dates, there is a change in the significance of the inbreeding effect reflecting a consistent trend in the data. At first, inbred fish grew more slowly than outbred fish; the proportional difference in sizes increased from 0.0% on day 6, up to day 20, when inbred fish were 10.8% smaller than outbred fish (figure 4). Subsequently, this proportional difference decreased until inbred fish were only 1.8% smaller on day 48 (figure 4). The relatively rapid growth of inbreds between days 20–48 may reflect the decreased density of inbred fish owing to mortality. The food treatment had a highly significant effect on final size, but the differences were small throughout the experiment. Low food fish were only 2.9% smaller at the end of the experiment than in the high food treatment.

### (e) *Timing of death as a result of partly deleterious factors*

Deaths as a result of partly deleterious, morphologically cryptic alleles occurred later than deaths caused by morphologically drastic recessive lethals (medians of day 16 and day 9, respectively; figure 5). This difference was highly significant (Mann–Whitney $U_{214,1088} = 23825$, $p < 0.0001$).

## 4. DISCUSSION

Our data demonstrate that, on top of *ca*. 1.5 early-acting, morphologically overt, completely penetrant recessive lethals per zygote (McCune *et al.* 2002), natural *D. rerio* are heterozygous for many other recessive deleterious alleles. These alleles cause substantial inbreeding depression of late larval and early juvenile viability. The increase of identity by descent from 0% to 25% leads to a 33% increase in mortality, from 9% in outbred fish to 42% in offspring from brother–sister mating. Mortality was the same under both food densities used in our experiments. Low food density was established by trial and error as the lowest food density which still does not increase mortality of outbred fish; apparently, this difference in food density does not affect inbreds, either.

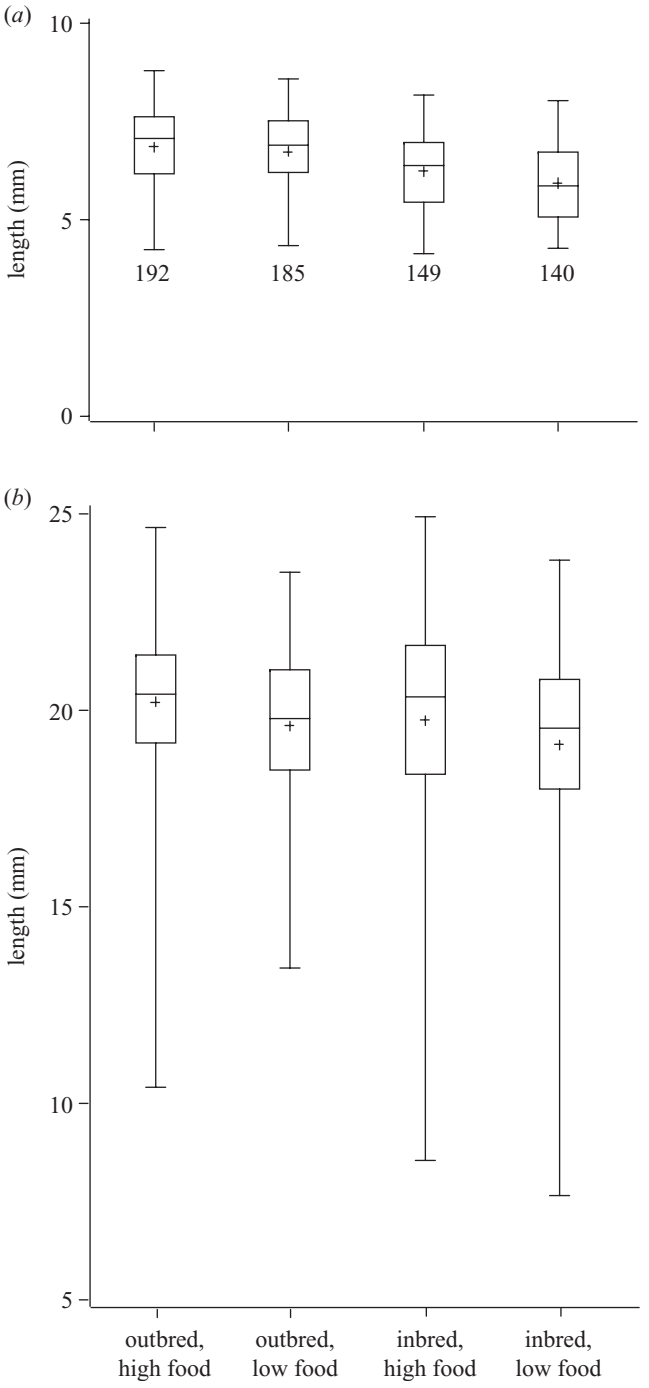


Figure 4. Box and whisker plots of length data for (*a*) 20 day fish, and (*b*) 48 day fish. The box represents 50% of the data, and whiskers show the range. The horizontal line within the box shows the median, whereas the plus symbol marks the mean. The numbers below the plots give the number of fish alive from the original 200 measured in that group on a given day. Note the higher mortality in inbred clutches on both days 20 and 48. As a result of this higher mortality, inbred fish were reared at a lower density during the latter part of the experiment.

However, food density affected growth rate (figure 4). The higher mortality of inbred fish leaves inbred survivors to grow under lower densities, which may have facilitated their subsequent growth, thus masking inbreeding depression in the growth rate. Initially, inbred fish grew slower than outbred fish (figure 4). At day 20, the average inbred fish was 10.8% shorter than an outbred one ($p = 0.025$). After this, fish density in tanks with inbred fish was substantially reduced (figure 4) and by day 48, the average inbred fish was only 1.8% shorter than an outbred one ($p < 0.64$).

Quantitatively, the observed inbreeding depression in viability implies 3.6 LEs of heterozygous recessive deleterious alleles which cause late larval and early juvenile mortality, per wild-caught individual. This number, based only on the genetic damage expressed upon inbreeding, *B*, is an underestimate, because the exact number should also include the component of genetic damage expressed in the outbred population (Morton *et al.* 1956). However, because mortality of outbred controls was only 9%, and some part of this background mortality must be environmental, the underestimation cannot be large. It is likely that in more stressful or complex environments, a larger number of LEs would be revealed (Jimenez *et al.* 1994; Kondrashov & Houle 1994; Keller & Waller 2002; Joron & Brakefield 2003).

Analysis of variance demonstrates that 20 $F_1$ fish from which the 10 $F_1$ brother–sister pairs were formed carry at least 17 different alleles that cause mortality in inbred offspring. Probably the real number is much higher. Indeed, all early-acting lethals detected previously in the parental fish (McCune *et al.* 2002) were unique, implying a high effective number of the wild source population. If later-acting deleterious alleles are also unique, 3.6 LEs per individual imply 72 different alleles. Even this may be an underestimation, because some later-acting alleles are probably not completely penetrant, i.e. do not always kill even homozygous individuals.

Taking into account early-acting lethals described in our earlier study, we conclude that in wild *D. rerio*, there are five LEs of deleterious alleles per zygote affecting viability at all stages before adulthood. This figure is in general agreement with estimates for humans and other vertebrates (see § 1), although exact comparison of data obtained by different authors is often impossible because (i) fitness was assayed under very different conditions, and (ii) different parts of the individual's life were studied. Obviously, it is difficult to study alleles which cause early prenatal mortality in mammals (see Shull & Neel 1972; Hussain 1998*a*; Kasarskis *et al.* 1998). By contrast, such alleles are easier to assay in fish and amphibians with transparent eggs. Very high estimates of the number of LEs in some birds (see Keller *et al.* 2002; Kruuk *et al.* 2002) were obtained in nature and include deleterious alleles affecting all the stages of life.

The most salient feature of late larval and early juvenile mortality of inbred fish is that it is morphologically cryptic. Not a single case of repeated morphological abnormality in dying individuals was observed (using 2.4× to 25× stereoscopic magnification) in this study, although a more careful scrutiny and behavioural study might reveal subtle abnormalities. Deleterious alleles that affect later stages of life (fecundity and longevity) are also likely to be morphologically cryptic (Slate *et al.* 2000). By contrast, embryonic and early larval mortality in *D. rerio* is always accompanied by abnormal morphology (McCune *et al.* 2002). Not a single one out of 55 early-acting lethals was morphologically cryptic, and most of them are apparently completely penetrant, i.e. always cause death of homozygous individuals. Thus, recessive deleterious

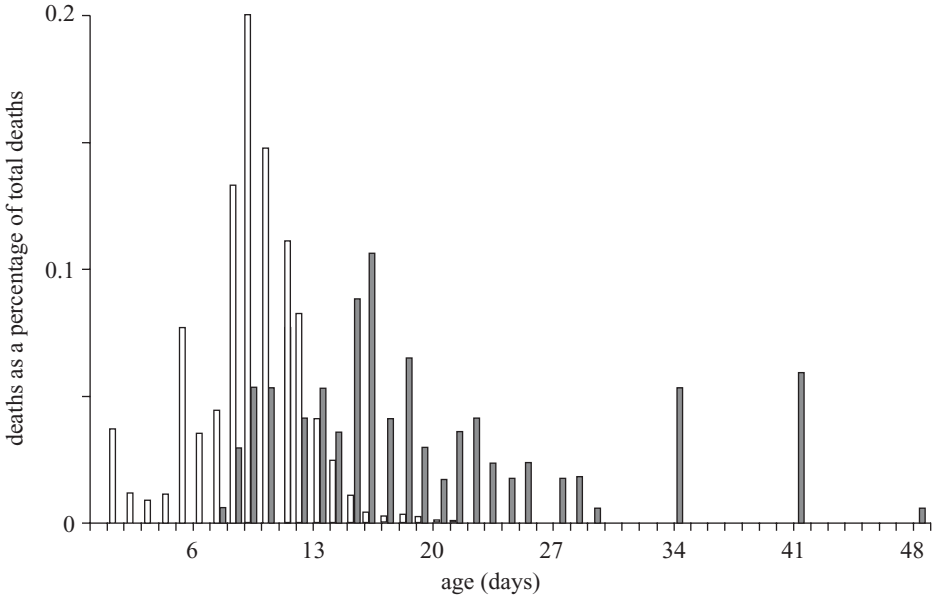


Figure 5. Age at death. Open bars show deaths of individuals homozygous for morphologically drastic, lethal recessives. Grey bars show deaths of inbred individuals not homozygous for recessive lethals; these deaths are presumably a result of the combined effects of multiple, morphologically cryptic, slightly deleterious alleles. Mortality data were recorded daily, up to day 30, and from then, on measurement sampling on days 34, 41 and 48. Day of death is earlier for individuals carrying drastic recessive lethals (Mann–Whitney $U_{214,1088} = 23825$, $p < 0.0001$).

Table 3. Contrasting features of the two classes of deleterious alleles.

| class 1 | class 2 |
|---|---|
| early acting | later acting |
| morphologically overt | morphologically cryptic |
| completely penetrant | incompletely penetrant |
| in homozygotes (lethal) | even in homozygotes |
| strongly recessive | partly dominant |

alleles in a natural population of *D. rerio* can be split into two rather distinct classes.

Extensive data on *Drosophila melanogaster* also reveal two distinct classes of naturally occurring deleterious alleles, which can be either recessively lethal (completely penetrant in homozygotes) or only slightly deleterious (low penetrance even in homozygotes), with rather few alleles with intermediate impacts on fitness (Timofeeff-Ressovsky 1935; Mukai *et al.* 1972; Lewontin 1974; Crow 1979). Most recessive lethals in *Drosophila* act during development, and imago lethals are very rare (see Hadorn 1961, p. 155). Later-acting deleterious alleles in flies are usually morphologically cryptic and mild (Timofeeff-Ressovsky 1935; Mukai *et al.* 1972). Lethals in *Drosophila* are much more recessive than mild alleles (Crow 1979).

It is very plausible that deleterious alleles in *D. melanogaster* and in *D. rerio* are subdivided into the same two classes. Combining what is known for these two species, we arrive at the list of features of these classes shown in table 3.

Of course, it would be desirable to study all these features in one species. In *Drosophila*, systematic data on morphological effects, if any, of naturally occurring recessive lethals are needed. So far, we only know that many recessive lethals in *Drosophila* cause clear-cut phenotypes (Nusslein-Volhard & Wieschaus 1980). In *D. rerio*, it is necessary to estimate, in addition to the number of LEs, the actual number of later-acting alleles per zygote, which, presumably, will demonstrate that many such alleles are mild. Such an estimate will require larger samples and more precise data on variance (Charlesworth & Hughes 2000; Rudan *et al.* 2003) than those obtained in our study. These data can also reveal heterozygous effects of later-acting deleterious alleles. However, we already know that early-acting lethals in *D. rerio* are strongly recessive: mortality was low among morphologically normal individuals even in those crosses where 2 out of 3 of such individuals must be heterogygous by a lethal (McCune *et al.* 2002). By contrast, later mortality of outbred controls was 9%, although a part of it was probably environmental.

The two classes of deleterious alleles may be a universal feature of all animals, or even all multicellular organisms. So far, only fragmentary data support this hypothesis. In particular, deleterious effects of inbreeding in humans are associated with obvious morphological phenotypes only in a few cases (Stoll *et al.* 1999; Zlotogora *et al.* 2003). Comprehensive analysis of the properties of spontaneous deleterious alleles may shed light on many genetic aspects of individual development.

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