STAGE-SPECIFIC SURVIVAL AND GENETIC VARIATION FOR RATES OF DEVELOPMENT IN THE ARGENTINIAN PEARL KILLIFISH *AUSTROLEBIAS BELLOTTI*.

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partial results on egg size variation, development rates and cox' proportional hazard models for mortality rates only

Introduction

Germ banks of dormant individuals have been found in many organisms. In evolutionary ecology, they are the battleground where the relevance of long-term fitness measures can be decided. Germ banks are often not very accessible for observation. Dormant stages are well protected, so that one sometimes can't even see whether a dormant embryo is still capable of hatching, or basically lost already. Annual killifish have the advantage of a transparent egg membrane, so that dormancy can be scored by monitoring the behaviour and development of the embryo. We combine an analysis of rates of development and mortality with an analysis of egg size variation and of hatching probability.

Material and Methods

The population was founded by eggs brought from Maschwitz, Argentina in 2004. These eggs were laid over two weeks by approximately sixty individuals (equal sex ratio) in four separate aquaria at a house in Maschwitz. In Leiden, individuals hatched from those eggs were used to start a pedigree population. The experiments are carried out by breeding the offspring of those. That implies that egg size variation is investigated in generation F2, and rates of development in F3, if the individuals collected in the wild are seen as P0.
Adults were kept in 35 liter plastic buckets. Each tank was used to house a pair of one male and one female. The buckets were placed in a greenhouse that was heated when the temperature dropped below 14 degrees and which was ventilated as soon as temperatures rose above 23 C degrees. Buckets contained tap water, some dead leaf material and one or two plants (*Elodea densa)*. Most of the tank surfaces were covered by duckweed (*Lemna minor*) which was scooped off regularly.
We collected up to six clutches from a male or a female. In between each collection, males and females were swapped, such that females had clutches with three different males and vice versa. In the first and third collection, there were brother-sister pairs in the breeding design.

During periods where we did not intend to collect eggs for the experiment, the tanks contained plastic two-liter containers with coconut peat where the adults could always lay eggs. In buckets housing pairs from which eggs were collected, a different container was placed the day before collection, which contained a mixture of glass beads and peat granules. The containers were left in the buckets for 24 hours, after which eggs

were collected. From the substrate, eggs can be sieved easily, and the granules increase the survival of eggs afterward (unpublished data). Sieving was done with tap water that was left to stand in the open for at least an hour with a bag of peat granules in it, to increase the acidity and to match the water to conditions in the containers. Once the eggs where collected, they were photographed (in water) together with a scale, using a binocular with a digital camera attached, and transferred to an incubator. Due to the morphology of the egg membrane, it was not possible to distinguish the perivitelline space and thus fertilized and unfertilized eggs immediately. At the day of collecting, adults were measured using digital calipers. Egg sizes were calculated by estimating their area relative to a scale on a digital photograph, using the program ImageJ. All together, 1149 eggs are included in the analysis, from 25 males and 24 females.

After being photographed, the eggs where placed on small wafers with 9 individual slots for eggs and a mesh bottom. Eggs from the same clutch were distributed over different wafers if necessary, such that wafer variation is nested within clutch variation. These wafers were hung in a tank with water that circulated past a UV lamp to keep it relatively clean, in a nearly dark growth chamber at 20 degrees. Embryos were incubated in 50% tap, 50% demi-water.

The experiment started on 27/02/2007 and eggs were collected until 26/04/2007. The eggs were submerged at all times and periodically monitored under a binocular. We classified development by assigning embryos to one of five stages (Table 1). Stage I covers pre-diapause I and diapause I stages (Wourms (1973). Diapause II occurs in stage III, diapause III in stage V.

On 01/07/2007, all wafers were transferred to boxes with wet vermiculite, to simulate dehydration of the ponds. Between 18 and 26/09/2007, we scored survival and checked whether eggs hatched. This last part of the experiment is a pilot for a future experiment where we will investigate genetic variation for development in "dry" conditions and hatching probabilities.

The data are analysed using (generalized) linear mixed models and mixed cox' proportional hazard models, including animal models. We used functions lmer and coxme in the R software package for that.

In the analysis of egg size variation, we allow for effects of both female and male on egg size. To assess genetic components of variation of these female and male contributions, we fitted four random effects, (1) for variation between fathers of females, (2) mothers of females, (3) fathers of males, and (4) mothers of males. If genetic variation for egg size follows a simple additive genetic model, the two variance components depending on the females should be of similar magnitude, and the two variance components which depend on the males as well. The number of days since 01/02/2007 is used as a covariate, as well as the sizes of the parents at the day the clutch was produced and clutch size. We did not fit random regression successfully. Models were compared using likelihood ratio tests, and parameter estimates assessed by inspecting posterior distributions simulated from the model.

For clutch sizes and hatching probabilities, we used generalized linear mixed models.

Development rates and survival are analysed using proportional hazards models with frailty (coxme). These frailties can be correlated as described by the kinship matrix, as in other animal models. By appropriate censoring, we managed to analyse both development and survival from the same data. When analysing survival rates, an observation is

considered censored if an individual makes a transition to the next developmental stage. In the analysis of development rates, observations are censored when individuals die.
In these analyses, the number of days since 01/02/2007 is used as a covariate, as well as the sizes of the parents at the day the clutch was produced, clutch size, and the inbreeding coefficient calculated from the kinship matrix.
Models with different covariates or random effects are compared using likelihood ratio tests, F-tests or t-tests. To assess the significance of genetic variances, likelihood intervals are used. We use 1.92 as the cutoff likelihood difference from the maximum likelihood to define interval boundaries (Pawitan).

Results

Egg size. Surprisingly, variance components representing genetic contributions to egg size variation did not follow a pattern according a simple additive genetic model. There was only significant variation found between the fathers of males and of females (Table two). That could indicate that genetic or environmental paternal effects play an important role in egg size variation and evolution. Egg size decreases slightly with the size of the female, clutch size has a negative effect on egg size, and later in spring eggs increase in size (Fig. 2).

Survival analysis. During wet storage, no individuals died while in stage five. Therefore, the survival analysis only comprises individuals in stages one to four. We find several significant covariates (Table 3). The estimate of the genetic variance for mortality rates is 0.11. We still have to assess significance of that variance component in detail. The software currently does not allow to estimate stage-specific genetic variances either. There is no significant interaction between relative egg size and stage, or between other covariates and stage. The stage effects of stages two to four are not significantly different. The implication is that survival differs between three stage groups: the first stage, stages two to four, and stage five. We also have to assess validity of the proportional hazard assumptions.

Development rates.
We find significant genetic variation in all rates (Table 4, Figures four and five).
Likelihood intervals (Fig. 4) indicate that the genetic variances of the different stages are not significantly different.
Different covariates are significant in the different stages.
We will impose censoring after 30 days, to investigate whether late events affect the results a lot.

Discussion

Acknowledgements

Martin Fourcade who allowed us to house the fish in Maschwitz. Kees Hofker and Rustin Ramadhin for help with the photos for Figure one. Terry Therneau for advice on the use of coxme.

Table 1. Developmental Stages

Stage		classification Wourms 1973
I	blastula and dispersed cell stages	1 – 28
II	somite formation	29 – 30
III	Long-somite/optic cup formation stages	31 - 36
IV	eyes become pigmented	37 -39
V	individuals become apparently ready to hatch	40 – 43

Table 2. Egg size variation

	Parameter estimate (s.e.)		Tail Probability (LR test) of parameter estimate
Fixed effects			
Intercept	2.123 (0.202)		
Female size	-0.017 (0.004)		< 0.001
Clutch size	-0.007 (0.001)		< 0.001
Date	0.002 (0.001)		0.001
Random effects			
sd of variation between fathers of males	0.460		< 0.001
sd of variation between fathers of females	0.106		< 0.001
Residual variation (sd)	0.209		

Table 3. Survival analysis

	Parameter estimate death rate (s.e.)		Tail Probability (Z test) of parameter estimate
Female size	0.070 (0.014)		< 0.001
Inbreeding coefficient	2.724 (0.619)		< 0.001
Date	-0.023 (0.004)		< 0.001
Stage two	-1.110 (0.232)		< 0.001
Stage three	-2.051 (0.455)		< 0.001
Stage four	-1.761 (0.364)		< 0.001
Random additive genetic variance	0.117		

Table 4. Rates of development

	Parameter estimate transition rate (s.e.)		Tail Probability z test
Stage one to two			
Clutch size	-0.050 (0.010)		< 0.001
Genetic variance	1.767		
Stage two to three			
Clutch size	-0.018 (0.009)		0.037*
Genetic variance	0.699		
Stage three to four			
Clutch size	-0.012 (0.004)		0.025*
Date	-0.020 (0.009)		0.0014*
Genetic variance	0.88		
Stage four to five			
Date	-0.031 (0.004)		<0.001*
Genetic variance	1.482		

Figure legends

Figure one. Photographs of stages scored

Figure two. Egg size depends on different covariates (female size, clutch size, date).

Figure three. Survivorship curves for embryos in different developmental stages. All embryos were stored in well-oxygenated water. Survival significantly differs between stages. Observations are censored when an individual proceeds to the next stage. Stages observed: (1) blastula/dispersed cell stage (includes diapause I); (2) somite formation; (3) long-somite/optic cup formation (diapause II); (4) eyes pigmented; (5) apparently ready to hatch (diapause III).

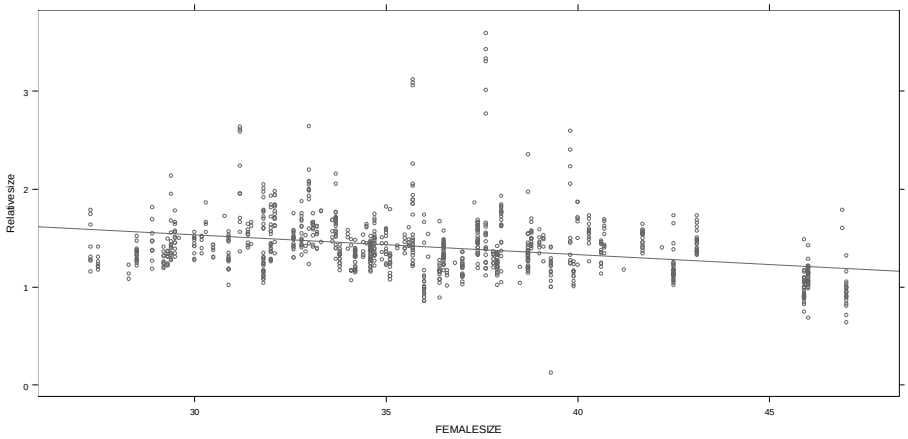
Figure four. (a) Graph of cumulative transition probabilities from 'dispersed cell' to the 'somite stage' beyond diapause I. Each curve is for a separate maternal grandmother family. In some families diapause sets in after approx. 35 days. There is significant genetic variation for rate of development. (b) (c) (d) Subsequent stages. Curves are again for separate maternal grandmother families.
The 'age in days' on the X-axis is the number of days individuals have spent already in the stage indicated on the Y-axis, the stage which they are developing from.

Figure five. Likelihood graphs genetic variances for development

Figure one

Add clear photos of the five stages and the range of variation within them

Figure two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35
40
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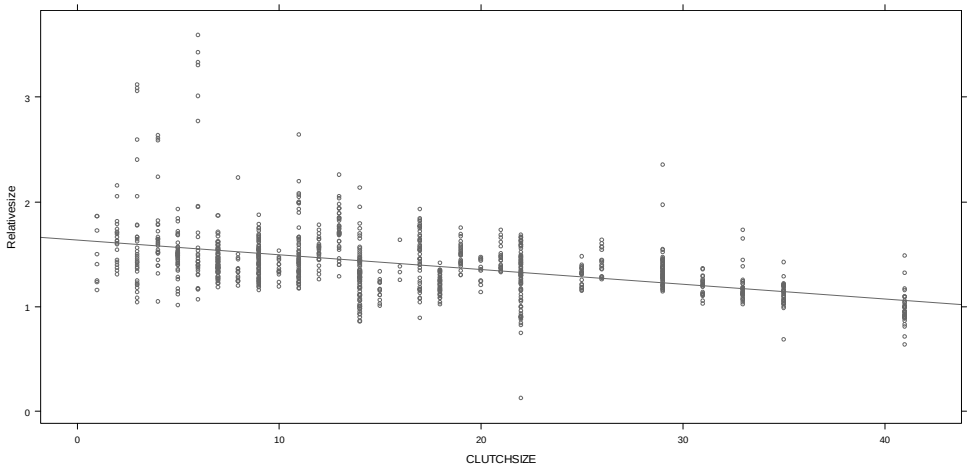
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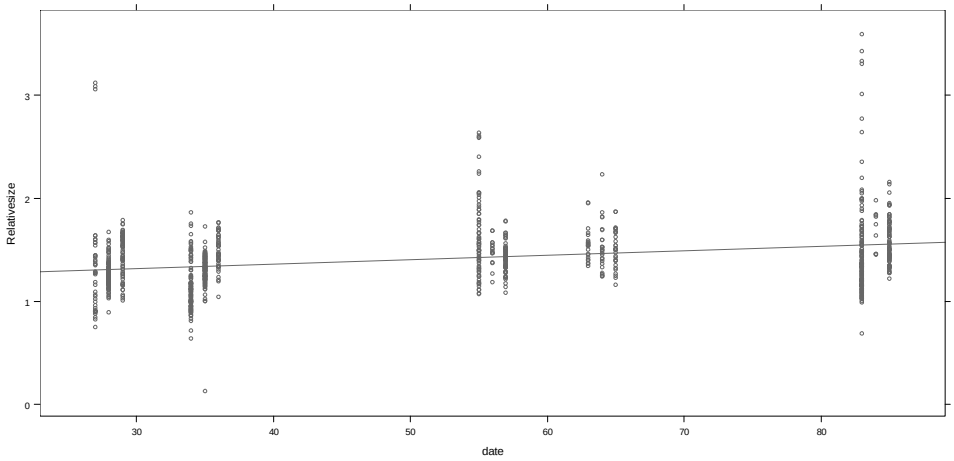
Relativesize
date
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1
2
3
30
40
50
60
70
80

Figure three

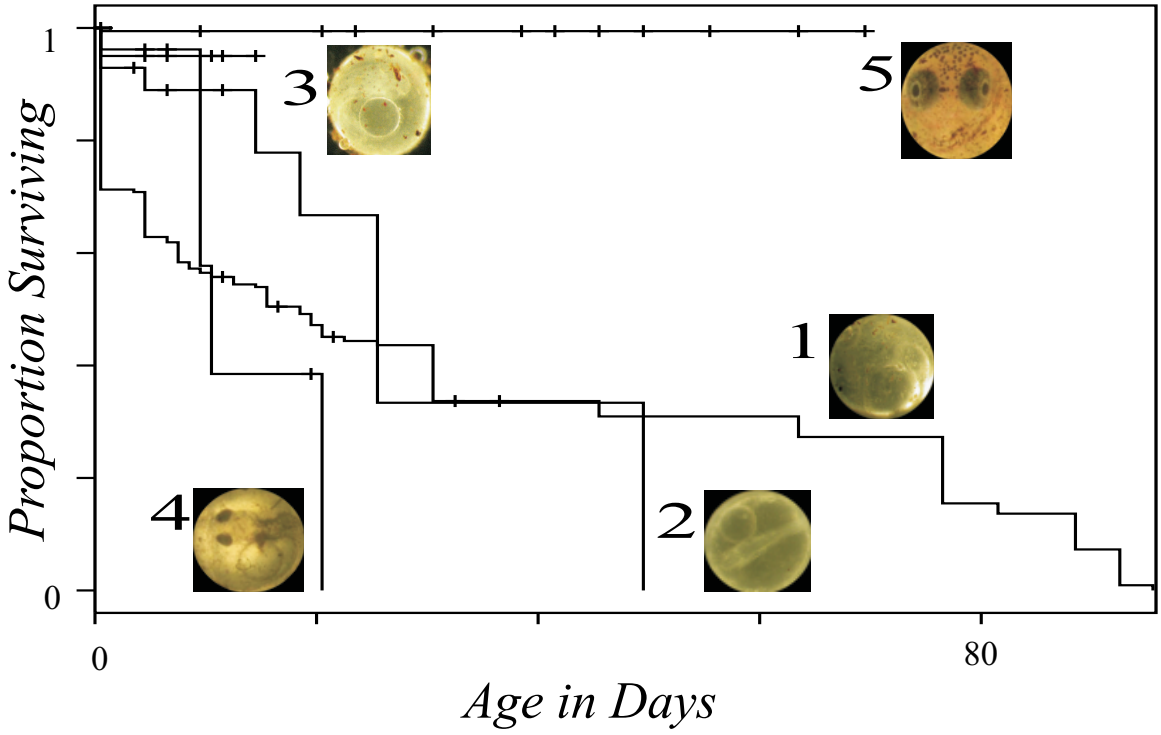
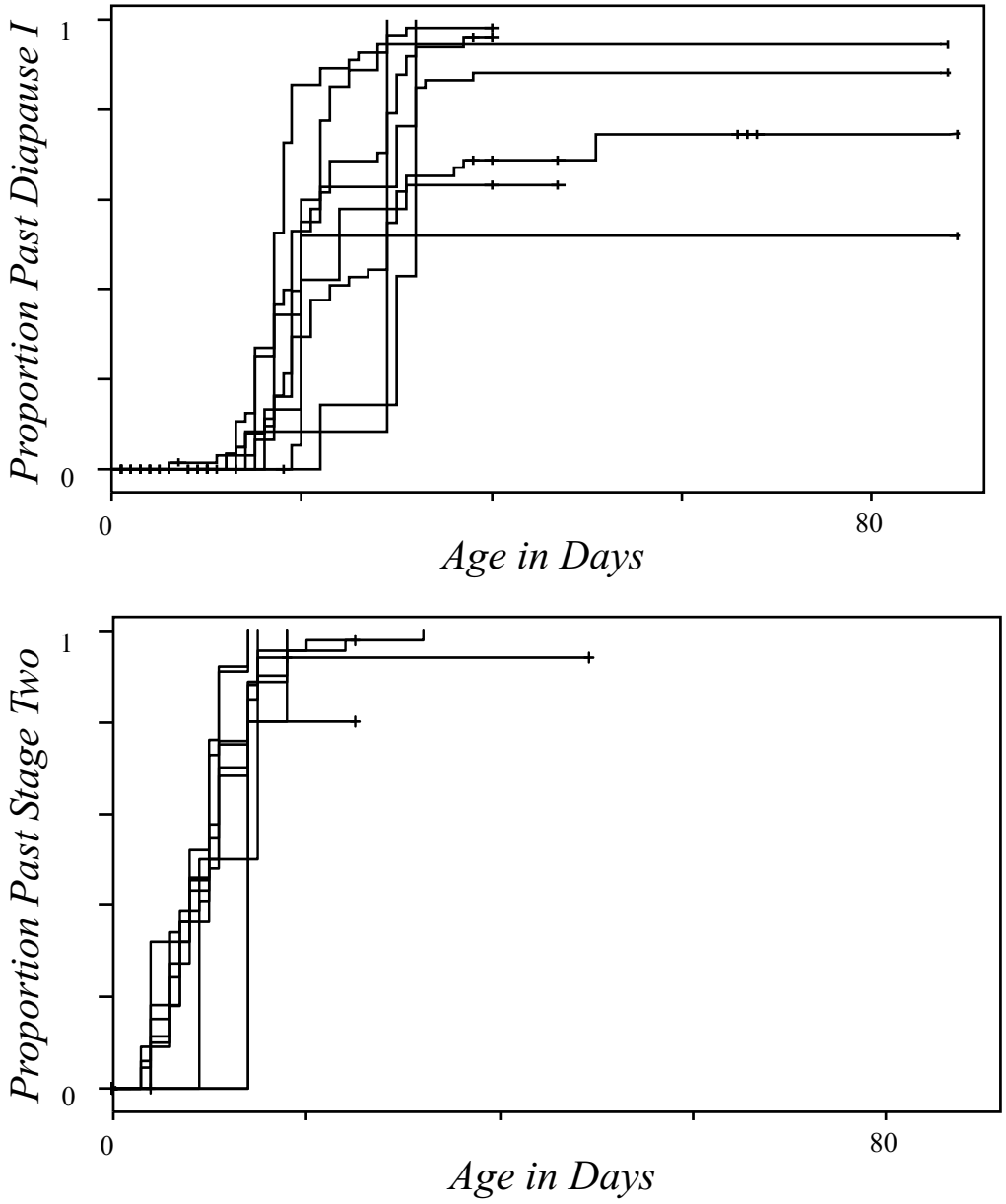
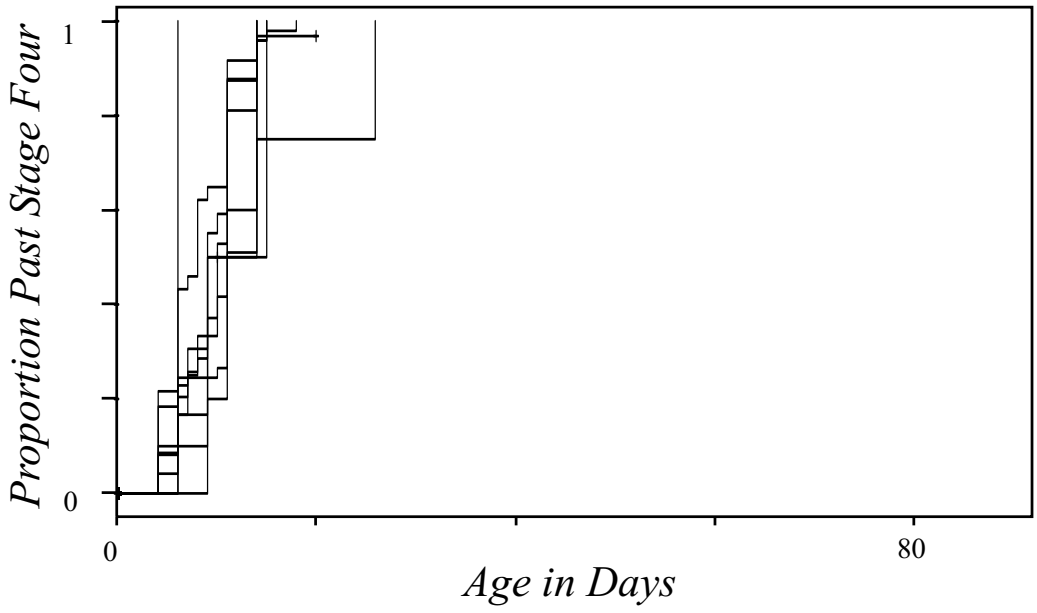


Figure three




Proportion Past Stage Three
1
0
0
80
Age in Days


Proportion Past Stage Four
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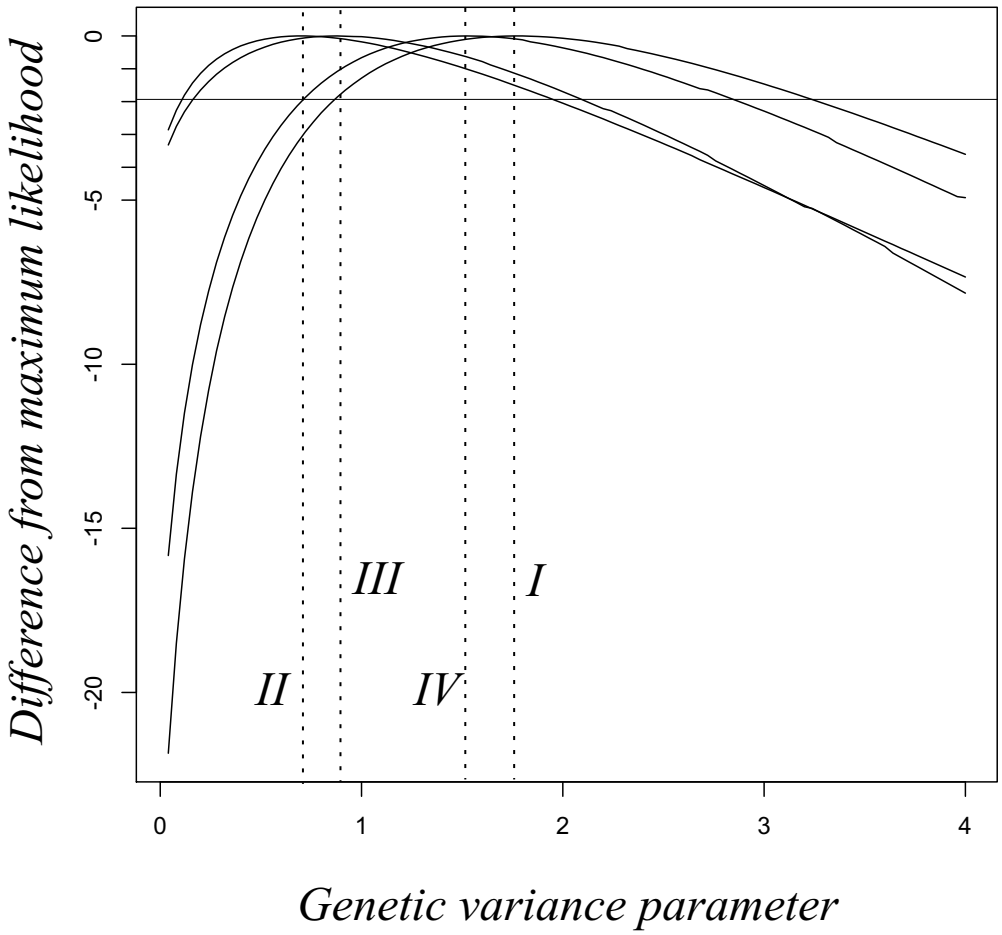
0
80
Age in Days

Figure four


Difference from maximum likelihood
0
-5
-10
-15
-20
0
1
2
3
4
II
III
IV
I
Genetic variance parameter