Climate change and pollution speed declines in zebrafish populations

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Table S1

Details of linear mixed effects (lme) models derived for developmental endpoints

In studies with hierarchical structure, as is often the case in toxicology, the error variance differs for each different level, whilst most parametric models assume only one error term. Another difficulty with hierarchical studies is the danger of pseudo-replication, either spatially or temporally. In this study the design includes hierarchy with different error variances, and spatial pseudo-replication, where fish from the same tank do not give independent data; the tank itself is a random effect. Mixed effects models, as generalised linear models, allow the best model to be fit to the collected data with the known parameters. In addition, the level of the data structure (e.g. repeated measure) is included as a random intercept, which means that the variance of the response can be separated into within- and between-subject variance components[1]. Linear Mixed Effects (LME) models allow complex designs thus we chose this model family to analyse our data (using R-statistics version 2.15.2, R Foundation for Statistical Computing).

S1.1 Specific growth rate

Mean specific growth rates (SGR_{weight} and SGR_{length}) per family and per treatment were compared for each of the degree day growth periods (Exposure days 0-14/15, 14/15-34/40 and 34/40-51/60). Fixed effects were period, breeding (inbred versus outbred families), clotrimazole exposure and temperature. Family was included as a random effect. The initial lme model included interactions between period, breeding and clotrimazole exposure and between breeding and temperature. The final best fit models were found to be additive models: SGR_{weight} ~ period + temperature (AIC = 2760); SGR_{length} ~ period + breeding + clotrimazole exposure + temperature (AIC = 1173).

S1.2 Sex ratio

Sex ratios were quantified at the end of the exposure study as the proportion of females in each family, per treatment. This took into account the effect of social interaction on sex determination/differentiation in each family, which were housed in separate tank compartments[2]. Proportions were transformed (arcsine, square root) before lme modelling. Fixed effects were breeding, clotrimazole exposure and temperature. Interactions (e.g. synergism) were considered initially between breeding and clotrimazole exposure and between breeding and temperature, but not between the two environmental factors, since their principal modes of action on sex determination (inhibition of aromatase) are the same and were assumed to be additive[3]. Family was included as a random effect. The final best fit model (AIC = 218) was an additive model including all three fixed effects: Arcsine square root proportion of females ~ breeding + clotrimazole exposure + temperature.

S1.3 Gonadal germ cell progression

Stage of gonadal development was defined in terms of germ cell progression for each individual (stage i, ii or iii) for each sex, family (tank compartment), and treatment. Fixed effects were gonadal sex, breeding, clotrimazole exposure and temperature. Individual animal was included as a random effect, in addition to family. The final best fit model (AIC = 993) was an additive model ~ gonadal sex + breeding.

S1.4 Gonad weight

Gonad weights (log_{10} transformed right gonad weights) were compared for each individual, for each sex, germ cell development stage, and family (tank compartment), per treatment using log_{10} body weight as a covariate. Fixed effects were log_{10} body weight, gonadal sex, gonadal stage, breeding, clotrimazole exposure, temperature. Individual animal was included as a random effect, in addition to family. The final best fit model (AIC = 570) was: log_{10} gonad weight ~ log_{10} body weight * gonadal sex * gonadal stage * breeding * clotrimazole exposure + temp. (* indicates a multiplicative interaction e.g. synergism, + indicates an additive interaction).

S1.5 Aromatase expression

Aromatase expression (log_{10} transformed *cyp19a1a* expression relative to the housekeeping gene *rpl8*) was compared in n=6 females from different families in each treatment. All females were at the same developmental stage – the most abundant gonadal germ cell development stage: i primary oocytes. Fixed effects were breeding, clotrimazole exposure and temperature. Family was included as a random effect. The initial and best fit model (AIC = 111) was: log_{10} aromatase expression ~ breeding * clotrimazole exposure + temperature. (* indicates a multiplicative interaction e.g. synergism, + indicates an additive interaction).

Table S1.1A: Linear mixed effects (lme) model of the influence of growth period, breeding, clotrimazole exposure and temperature on specific growth rate based on wet weight (SGR_{weight})

Parameter	Initial model (AIC = 2763)			Best fit model (AIC = 2760)		
	df	t-value	*p*-value	df	t-value	*p*-value
(Intercept)	516	18.740842	0.0000	525	44.21295	0.0000
Growth period (1/2/3)	516	-14.949220	**0.0000**	525	-39.47455	**0.0000**
Breeding (in/out)	36	2.198038	0.0345			
Clotrimazole (low/control)	516	2.571851	**0.0104**			
Clotrimazole (high/control)	516	1.522590	0.1285			
Temperature (28/33°C)	516	3.696080	**0.0002**	525	4.45007	**0.0000**
Growth period×breeding	516	-1.786088	0.0747			
Growth period×low clotrimazole	516	-2.191622	0.0289			
Growth period×high clotrimazole	516	-1.184986	0.2366			
Breeding×low clotrimazole	516	-0.942814	0.3462			
Breeding×high clotrimazole	516	-0.268396	0.7885			
Growth period×breeding×low clotrimazole	516	0.827256	0.4085			
Growth period×breeding×high clotrimazole	516	0.288040	0.7734			

Significant effects in **bold** typeface

Initial model: fixed effects on SGR_{wt} ~ growth period * breeding * dose + temperature; random effect = family.

Best fit model: fixed effects: SGR_{wt} ~ growth period + temperature; random effect = family.

All fixed effects are categorical variables. Random effect is family (inbred 1-18, outbred 1-20).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Table S1.1B: Linear mixed effects (lme) model of the influence of growth period, breeding, clotrimazole exposure and temperature on specific growth rate based on standard length (SGR_{length})

Parameter	Initial model (AIC = 1189)			Best fit model (AIC = 1173)		
	df	t-value	*p*-value	df	t-value	*p*-value
(Intercept)	516	22.124920	0.0000	522	33.68445	0.0000
Growth period (1/2/3)	516	-16.841512	**0.0000**	522	-28.26626	**0.0000**
Breeding (in/out)	36	1.955093	0.0584	36	1.58791	0.1211
Clotrimazole (low/control)	516	2.994716	**0.0029**	522	0.83895	0.4019
Clotrimazole (high/control)	516	0.576241	0.5647	522	0.42345	0.6721
Temperature (28/33°C)	516	2.449853	**0.0146**	522	2.44249	**0.0149**
Growth period×breeding	516	-1.526349	0.1275	522	-1.15786	0.2475
Growth period×low clotrimazole	516	-2.688911	0.0074			
Growth period×high clotrimazole	516	-0.401908	0.6879			
Breeding×low clotrimazole	516	-1.586510	0.1132			
Breeding×high clotrimazole	516	-0.658711	0.5104			
Growth period×breeding×low clotrimazole	516	1.288006	0.1983			
Growth period×breeding×high clotrimazole	516	0.573846	0.5663			

Significant effects in **bold** typeface

Initial model: fixed effects on SGR_{lth} ~ period * breeding * dose + temperature; random effect = family.

Best fit model: fixed effects on SGR_{lth} ~ period * breeding + dose + temperature; random effect = family.

All fixed effects are categorical variables. Random effect is family (inbred 1-18, outbred 1-20).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Table S1.2: Linear mixed effects (lme) model of the influence of breeding, clotrimazole exposure and temperature on sex ratio (arcsine square root proportion of females per family per treatment)

Parameter	Initial model (AIC = 224)			Best fit model (AIC = 218)		
	df	t-value	*p*-value	df	t-value	*p*-value
(Intercept)	146	8.616649	0.0000	148	9.416995	0.0000
Breeding (in/out)	36	3.038948	**0.0044**	36	4.477125	**0.0001**
Clotrimazole (low/control)	146	-1.182211	0.2390	148	-0.413375	0.6799
Clotrimazole (high/control)	146	-4.192633	**0.0000**	148	-6.276231	**0.0000**
Temperature (28/33°C)	146	-3.680676	**0.0003**	148	-3.676509	**0.0003**
Breeding×low clotrimazole	146	1.282888	0.2016			
Breeding×high clotrimazole	146	-0.181759	0.8560			

Significant effects in **bold** typeface

Initial model: fixed effects on arcsine sqrt proportion of females ~ breeding * clotrimazole exposure + temperature; random effect = family.

Best fit model: fixed effects on arcsine sqrt proportion of females ~ breeding + clotrimazole exposure + temperature; random effect = family.

All fixed effects are categorical variables. Random effect is family (inbred 1-18, outbred 1-20).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Table S1.3: Linear mixed effects (lme) model of the influence of log_{10} body weight, gonadal sex, stage, breeding, clotrimazole exposure and temperature on log_{10} gonad weight

Parameter	Initial and best fit model (AIC = 570)		
	df	t-value	*p*-value
(Intercept)	297	-2.7159275	0.0078
Log_body_weight	297	-0.3254790	0.7450
Sex (M/F)	297	2.5887624	**0.0101**
Stage (1/2/3)	297	1.4120575	0.1590
Breeding (in/out)	35	0.5561966	0.5816
Clotrimazole (low/control)	297	0.3156156	0.7525
Clotrimazole (high/control)	297	-0.0982313	0.9218
Temperature (28/33°C)	297	0.0063095	0.9950
Log_body_weight:sex	297	2.4795349	**0.0137**
Log_body_weight:stage	297	1.2584813	0.2092
Sex:stage	297	-2.6512701	**0.0084**
Log_body_weight:breeding	297	0.6595118	0.5101
Sex:breeding	297	-0.0556987	0.9556
Stage:breeding	297	-0.3243966	0.7459
Log_body_weight:low clot	297	0.3798591	0.7043
Log_body_weight:high clot	297	-0.0575429	0.9542
Sex:low clotrimazole	297	-0.9670904	0.3343
Sex:high clotrimazole	297	-0.6122394	0.5408
Stage:low clotrimazole	297	-0.8748545	0.3824
Stage:high clotrimazole	297	0.0371107	0.9704
Breeding:low clotrimazole	297	-0.0945798	0.9247
Breeding:high clotrimazole	297	1.1255811	0.2613
Log_bodywt:sex:stage	297	-2.4506156	**0.0148**
Log_bodywt:sex:breeding	297	-0.0154394	0.9877
Log_bodywt:stage:breeding	297	-0.4869563	0.6266
Sex:stage:breeding	297	-0.0642231	0.9488
Log_bodywt:sex:low clot	297	-0.9686878	0.3335
Log_bodywt:sex:high clot	297	-0.6088119	0.5431
Log_bodywt:stage:low clot	297	-1.0077234	0.3144
Log_bodywt:stage:high clot	297	-0.1124103	0.9106
Sex:stage:low clot	297	1.4991316	0.1349

Sex:stage:high clot	297	0.4491426	0.6537
Log_bodywt:breed:low clot	297	-0.1732852	0.8625
Log_bodywt:bred:high clot	297	1.0757006	0.2829
Sex:breed:low clotrimazole	297	-0.3640634	0.7161
Sex:breed:high clotrimazole	297	0.0743347	0.9408
Stage:breed:low clotrimazole	297	0.1563683	0.8758
Stage:breed:high clotrimazole	297	-1.1824296	0.2380
Log_bodywt:sex:stage:breed	297	-0.0653231	0.9480
Log_bodywt:sex:stage:high clot	297	1.5788938	0.1154
Log_bodywt:sex:stage:high clot	297	0.5405424	0.5892
Log_bodywt:sex:breed:low clot	297	-0.3703319	0.7114
Log_bodywt:sex:breed:high clot	297	-0.1692549	0.8657
Log_bodywt:stage:breed:low clot	297	0.2780639	0.7812
Log_bodywt:stage:breed:high clot	297	-1.0205510	0.3083
Sex:stage:breeding:low clot	297	0.2381292	0.8119
Sex:stage:breeding:low clot	297	0.3433032	0.7316
Log_bodywt:sex:stage:breed:low clot	297	0.1841107	0.8541
Log_bodywt:sex:stage:breed:high clot	297	0.4933085	0.6222

Significant effects in **bold** typeface

Initial and best fit model: fixed effects on log_gonad_weight ~ log_body_weight * gonad_sex * gonad_stage* breeding * dose + temperature; random effects = family and individual.

All fixed effects are categorical variables except the covariate log_body_weight. Random effects are family (inbred 1-18, outbred 1-20) and individual (1-4).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Table S1.4: Linear mixed effects (lme) model of the influence of gonadal sex, breeding, clotrimazole exposure and temperature on germ cell progression

Parameter	Initial model (AIC = 1010)			Best fit model (AIC = 993)		
	df	t-value	*p*-value	df	t-value	*p*-value
(Intercept)	500	16.712741	0.0000	509	21.38003	0.0000
Sex (M/F)	500	14.379486	**0.0000**	509	32.28709	**0.0000**
Breeding (in/out)	36	-0.406454	0.6868	36	3.14891	**0.0017**
Clotrimazole (low/control)	500	-0.296678	0.7668			
Clotrimazole (high/control)	500	-0.242949	0.8081			
Temperature (28/33°C)	500	-0.334590	0.7381	509	-1.56436	0.1184
Sex×breeding	500	1.052747	0.2930			
Sex×low clotrimazole	500	-1.741223	0.0823			
Sex×high clotrimazole	500	-0.127810	0.8948			
Breeding×low clotrimazole	500	0.832651	0.4045			
Breeding×high clotrimazole	500	0.482947	0.6293			
Sex×breeding×low clotrimazole	500	0.381902	0.7027			
Sex×breeding×high clotrimazole	500	-0.040515	0.9677			

Significant effects in **bold** typeface

Initial model: fixed effects on germ cell progression ~ gonadal sex * breeding * clotrimazole exposure + temperature; random effects = family and individual.

Best fit model: fixed effects on germ cell progression ~ gonadal sex + breeding, random effects = family and individual.

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

All fixed effects are categorical variables. Random effects are family (inbred 1-18, outbred 1-20) and individual (1-4).

Table S1.5: Linear mixed effects (lme) model of the influence of breeding, clotrimazole exposure and temperature on ovarian aromatase expression (log_{10} transformed)

Parameter	Initial and best fit model (AIC = 111)		
	df	t-value	*p*-value
(Intercept)	25	-4.505143	0.0001
Breeding (in/out)	21	0.004159	0.9967
Clotrimazole (low/control)	25	-1.308236	0.2027
Clotrimazole (high/control)	25	4.202001	**0.0003**
Temperature (28/33°C)	25	1.543240	0.1353
Breeding×low clotrimazole	25	0.251999	0.8031
Breeding×high clotrimazole	25	-2.620412	**0.0147**

Significant effects in **bold** typeface

Initial and best fit model: fixed effects on log_{10} aromatase expression ~ breeding * clotrimazole exposure + temperature; random effect = family.

All fixed effects are categorical variables. Random effect is family (inbred 1-18, outbred 1-20).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Table S2

Details of the parental pair breeding applied to generate the F_3 inbred and outbred lines

Table S2.1: Outbreeding applied to generate F_1 fish (grandparents of fish used in final study)

Outbred population/ individual male	Outbred population/ individual female	F_1 family ID
1/1	2/1	1
2/2	1/2	2
1/3	2/3	3
2/4	1/4	4
1/5	2/5	NE
2/6	1/6	6
1/7	2/7	7
2/8	1/8	8
1/9	2/9	9
2/10	1/10	10
1/11	2/11	11
2/12	1/12	12
1/13	2/13	13
2/14	1/14	NE
1/15	2/15	15
2/16	1/16	16
1/17	2/17	17
2/18	1/18	18
1/19	2/19	19
2/20	1/20	20
1/21	2/21	21
2/22	1/22	22
1/23	2/23	NE
2/24	1/24	24

Population 1 = Mozahadi, Gastala Bazar, Tarakanda, 10 km north of Mymensingh and the Brahmaputra River (Latitude 24.8710109 Longitude 90.4148744). Population 2 = Kechuri Beel, Badai Barera, Kotwali, Mymensingh, adjacent to the Brahmaputra River (Lat 23.4067115, Long 88.4979698). All individuals were sampled randomly from each population (n=100).
NE = No embryos (from non viable or non compatible pair)

Table S2.2: Outbreeding applied to generate F_2 fish (parents of fish used in final study)

Outbred Family[#] (males)	Outbred Family[#] (females)	F_2 family ID
1	2	A
3	4	B
6	7	C
8	9	D
10	11	E
12	13	F
15	16	G
17	18	H
19	20	I

21	22	J
2	3	K
4	6	L
7	8	M
24	10	N
11	12	O
13	15	P
16	17	Q
18	19	R
20	21	S
22	1	T

[#] Families are full-sibling families and correspond to family IDs in Table S2.1

Table S2.3: Outbreeding applied to generate F_3 test fish (via reciprocal crossing)

Outbred Family[†] (males)	Outbred Family[†] (females)	Outbred F_3 family ID
A	B	OP1
B	A	OP2
C	D	OP3
D	C	OP4
E	F	OP5
F	E	OP6
G	H	OP7
H	G	OP8
I	J	OP9
J	I	OP10
K	L	OP11
L	K	OP12
M	N	OP13
N	M	OP14
O	P	OP15
P	O	OP16
Q	R	OP17
R	Q	OP18
S	T	OP19
T	S	OP20

Note [†] Families are full sibling families and correspond to family IDs in Table S2.2

Table S2.4: Inbreeding applied to generate F3 test fish

Outbred Family[*] (males)	Outbred Family[*] (females)	Inbred F_3 family ID
A	A	IP1
B	B	IP2
C	C	IP3
D	D	IP4
E	E	IP5

F	F	IP6
G	G	IP7
H	H	IP8
I	I	IP9
J	J	IP10
K	K	IP11
L	L	IP12
M	M	IP13
N	N	IP14
O	O	IP15
P	P	IP16
Q	Q	IP17
R	R	IP18
S	S	IP19
T	T	IP20

* Families are full-sibling families and correspond to family IDs in Table S2.2

F_3 families denoted 1-20 (for inbreds I and outbreds O)

F_2 families denoted (A-T rather than 1-20)

The family-level replication and the degree and rate of inbreeding (one generation of full-sibling mating) were consistent with those used in other studies assessing for inbreeding effects[4,5,6]. Our fish were the great grandchildren of wild (Bangladesh origin) male and female zebrafish (F_0) (see Fig. S4). The approach used is consistent with the practice of out breeding between strains, which is performed routinely in animal husbandry[7]. Introgression of individuals from wild populations has also been advocated in order to maintain representative outbred stocks for use in ecotoxicology[8,9]. Such practices can sometimes lead to outbreeding depression in F_1 and/or F_2 generations[10,11] due to a break-up of favourable epistatic interactions in the parental lines, or phenotype-environment interaction[11,12,13]. The use of F_3 generation hybrids in our study minimised the possibility of outbreeding depression.

Table S3

Water quality parameters

The following water quality parameters were monitored throughout the exposure study and measurements were within guideline limits for fish sexual development tests[14]:

Dissolved oxygen 70-100% saturation

pH 7.1-8.2

Total ammonia-N <2 μg/L

Chlorine <2 μg/L

Water hardness 41-69 mg/L

Alkalinity 21-39 mg/L

Suspended solids <3.00 mg/L

Total Organic Carbon <1-2.5 mg/L

Chemical Oxygen Demand <10 mg/L

Inorganic and organic analytes <predicted no effect concentrations.

Table S4

S4.1 Concentrations of clotrimazole (µg/L) in samples of aquaria water measured by LC-MS throughout the *in vivo* exposure study

Exposure day	Control (0 µg/L nominal)			Low-level clotrimazole exposure (2 µg/L nominal)			High-level clotrimazole exposure (10 µg/L nominal)		
	Mean	St.dev.	n	Mean	St.dev.	n	Mean	St.dev.	n
-4	0^{c}	0	3	0.48^{d}	0.01	3	2.9^{c}	0.66	3
	0^{d}	0	1	0.45^{c}	-	1	3.1^{d}	-	1
0	0^{b}	0	3	0.71^{d}	0.025	3	3.1^{a}	0.12	3
	0^{a}	0	1	-	-	-	3.3^{c}	5.44E-16	3
4	0^{a}	0	3	0.94^{c}	0.222	3	6.07^{c}	0.15	3
	0^{b}	0	1	1.5^{d}	-	1	5.7^{d}	-	1
9	0^{c}	0	3	2.1^{d}	0.058	3	6^{a}	0.1	3
	0^{d}	0	1	-	-	-	5.9^{c}	-	1
15	0^{c}	0	3	1.6^{c}	2.72E-16	3	7.1^{a}	0.1	3
	0^{d}	0	1	1.7^{d}	-	1	7.3^{d}	0.2	3
17	0^{b}	0	3	2.0^{d}	0.025	3	11.7^{b}	0.46	3
	0^{c}	0	1	2.1^{c}	-	1	11.3^{c}	0.58	3
35	0^{d}	0	3	1.57^{d}	0.05	3	9.3^{a}	0.15	3
	0^{a}	0	1	1.5^{c}	-	1	9.3^{c}	0.15	3
56	0^{a}	0	3	2.4^{d}	0.21	3	8.67^{a}	0.21	3
	0^{b}	0	1	2.7^{c}	-	1	8.03^{d}	0.55	3
77	0^{d}	0	3	2.1^{c}	0.058	3	8.8^{b}	0.2	3
	0^{c}	0	1	2.2^{d}	-	1	10.1^{c}	0.25	3
119	0^{b}	0	3	1.7^{c}	0.05	3	12^{a}	0	3
	0^{c}	0	1	1.5^{d}	-	1	10^{d}	0	3
161	0^{b}	0	3	3.6^{c}	0.17	3	11.5^{c}	0.87	3
	0^{a}	0	1	3.2^{d}	-	1	11.3^{b}	0.58	3
178	0^{d}	0	3	1.5^{d}	0.058	3	9.2^{d}	0.2	3
	0^{c}	0	1	1.5^{c}	-	1	9.2^{c}	-	1

Note superscripts: ^{a} = Inbred 28°C; ^{b} = Outbred 28°C; ^{c} = Inbred 33°C; ^{d} = Outbred 33°C.

See Table S4.2 for the mean measured concentrations of clotrimazole.

Water sampling: Water sampling for analysis of clotrimazole was conducted at twelve time points during the study (exposure days -4, 0, 4, 9, 15, 17, 35, 56, 77, 119, 161, 178) in aquaria representing all treatment combinations (Table 1 in manuscript), including all three clotrimazole exposure concentrations (0 µg/L, 2 µg/L, 10 µg/L nominal concentrations) at every time point. On each occasion two tanks were selected randomly for each treatment combination, and water samples were taken either in triplicate (to assess consistency in water sampling and analysis), or as single samples.

The number of water samples taken for analysis from the different treatment regimes during the study, and the variation (standard deviation) for clotrimazole measurements for samples measured in triplicate are shown in Table S4.1. Chemical analysis was performed on the same day as water sampling.

Quantification by LC-MS: Initial chromatographic separation of clotrimazole was carried out on a Gemini-NX C18 column (50 x 2 mm, 3.0 µm, Phenomenex, Torrance, CA). The column was fitted with a pre-filter (0.5 µm, Supelco, USA) maintained at 50°C and the flow rate was 500 µl/min. The elution gradient of eluent was A) 0.1% ammonium hydroxide in water and eluent B) LCMS grade methanol (T(min)/ % A was 0/90→3/0→5/0→5.1/90→6/90). A Quadrupole Ion Trap (Thermo-Finnigan TSQ Quantum Access) mass spectrometer with electrospray ionisation was used with the following parameters: sheath gas flow 60 arbitrary units, auxiliary gas flow 50 arbitrary units, spray voltage 3.0 kV, capillary temperature 300°C, capillary offset voltage 39 V, tube lens offset tuned. Positive ionization with selected reaction monitoring (SRM) was used for all analyses. The analyte (clotrimazole) corresponded to a product ion mass of 169 Da and the limit of quantitation was 0.2 µg/L. Analytical standards and spiked chemical recoveries were consistently shown to be >95% of nominal.

S4.2 Overall mean concentrations of clotrimazole (µg/L) in aquaria water measured by LC-MS throughout the *in vivo* exposure study

Exposure day	Control (0 µg/L nominal)	Low-level clotrimazole exposure (2 µg/L nominal)	High-level clotrimazole exposure (10 µg/L nominal)
-4	0	0.47	2.94
0	0	0.71	3.2
4	0	1.2	5.9
9	0	2.13	6.0
15	0	1.65	7.24
17	0	2.03	11.5
35	0	1.53	9.33
56	0	2.6	8.35
77	0	2.13	9.45
119	0	1.6	10.9
161	0	3.4	11.4
178	0	1.5	9.2
Arithmetic mean	**0**	**1.9**	**8.4**
Geometric mean	**0**	**1.73**	**7.95**
95% CI	0	0.42	1.54
SEM	0	0.22	0.79
Limit of detection	0.2	0.2	0.2

Table S5

Histological processing of gonad tissues

Whole bodies were fixed (maximum 6 hours) in Bouin's solution (Sigma Aldrich, Dorset, UK), and subsequently progressively dehydrated in 70-100% industrial methylated spirits and then embedded in paraffin wax for histopathology. Serial transverse body sections (replicate 5 µm sections, obtained at four 500-1000 µm intervals) were mounted on glass slides, stained using haematoxylin and eosin and examined using a Leitz Diaplan (Leica Microsystems GmbH, Wetzlar, Germany) light microscope (×10-100) magnification). The most mature germ cell stages were recorded in individual male gonads (stages: i spermatogonia; ii spermatocytes; iii spermatids/spermatozoa) and individual female gonads (stages: i primary oocytes; ii cortical alveolar/secondary oocytes; iii vitellogenic oocytes) as a measure of the progression of gonadal development.

Table S6

Primers and conditions for q-PCR analysis of target genes

Target gene	Forward primer reading from 5' to 3'	Reverse primer reading from 5' to 3'	Annealing temp (°C)	Efficiency (%)
Ribosomal protein l8 (*rpl8*) NCBI accession NM_00131154.2	CCG AGA CCA AGA AAT CCA GAG	CCA GCA ACA ACA CCA ACA AC	59.5	2.07
Aromatase (*cyp19a1a*) NCBI accession NM_200713.1	AGC CGT CCA GCC TCA G	ATC CAA AAG CAG AAG CAG TAG	61.5	1.89

Total mRNA was extracted from each gonad tissue sample using RNeasy micro-kits (Qiagen, Crawley, West Sussex, UK), following the manufacturer' instructions. Total mRNA concentration was estimated from absorbance at 260 nm using a Nanodrop spectrophotomer (Thermo Finnigan, Hemel Hempstead, UK) and RNA quality was verified by absorbance ratios 260 nm/280 nm >1.8. Following DNase enzyme treatment, cDNA was reverse transcribed from the pure mRNA extracts using "Superscript Vilo®" (Invitrogen, Paisley, UK) according to the manufacturer's instructions.

Oligonucleotide primer pairs (forward and reverse) were designed using Beacon Designer 3.0 software (Premier Biosoft International, Palo Alto, CA) and purchased from Invitrogen. Primer-pair annealing temperatures were optimized for real-time PCR on a temperature-gradient program. Primer specificity was confirmed by gel electrophoresis and/or melt curve analysis and automated fluorescence sequencing of PCR products. To determine the detection range, linearity and real-time PCR amplification efficiency (E; $E = 10[-1/\text{slope}]$)[15] of each primer pair, real-time PCR amplifications were run in triplicate on a 10-fold serial dilution series of zebrafish gonad cDNA pooled from all testis and ovary samples, respectively, and standard curves were calculated referring the threshold cycle (Ct; the PCR cycle at which fluorescence increased above background levels) to the logarithm of the cDNA dilution.

During real-time PCR each cDNA sample was amplified in triplicate using 96-well optical plates in a 20-µl reaction volume using 1 µl cDNA, 10 µl 2× Absolute SYBR Green (Flourescein) Supermix (BioRad), 5 µM of the appropriate forward and reverse primers. Hot start Taq polymerase was activated by an initial denaturation step at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at the primer-specified temperatures for 20 sec and, finally, melt curve analysis. All samples were run on the same plate ensuring consistent quantification of the expression of each target gene.

Relative expression levels were determined using the following calculation:

$$RE = (E\ \text{ref})^{Ct\ \text{ref}} / (E\ \text{target})^{Ct\ \text{target}}$$

Where *RE* is relative gene expression, *ref* is the housekeeping gene, *target* is the gene of interest, *E* is PCR amplification efficiency and *Ct* is cycle threshold (number of temperature cycles yielding above background expression) for that particular gene.

Table S7

Parameterisation of the Vortex PVA model for zebrafish

Parameter	Inbred population		Outbred population	
	Mean	SD	Mean	SD
Juvenile survivorship % (age 0+)	4	3	9	3
Adult survivorship %[16] (age 1+, 2+)[17]	19	3	19	3
Fecundity[16,17] (age 1+, 2+)	50	20	50	20
Carrying capacity K[16]	5000	1000	5000	1000
Lethal equivalents LE	5		5	
Sex ratio (% females)	3-41%		17-58%	

PVA model software: Vortex©, version 9.99c[18,19].

Parameterisation: Control models for unperturbed (non exposed) inbred and outbred zebrafish populations were parameterised based on data from our (pre)exposure study quantifying survival and fecundity for inbred and outbred families and wider published life-history data for wild zebrafish[16,17,20-24] .

Demographics: Populations were assumed to be "closed", consisting initially of 4000 individuals. Asymptotic population growth was modelled using a logistic model[19], adopting a ceiling carrying capacity e.g. K = 5000 ± 1000 total individuals initially, rather than functional forms of density dependence. Upon reaching stable age distributions, individuals were divided between two age classes (*circa* 3800-3940 age 0+ juveniles and 60-200 age 1+ adults). Age distributions were verified against observed adult population counts in natural ponds[16].

Age 0+ survivorship was 4 ± 3% for inbreds and was 9 ± 3% for outbreds. Age 1+ and 2+ survivorship was 19 ± 3% for both inbreds and outbreds. There was assumed to be no difference in male and female survivorship and this was based on limited sexual size dimorphism in zebrafish[20,25]. Breeding was limited to 60 annual spawning events per adult female in their second year (age 1+), and when applicable, their third year (age 2+), simulating a mean inter-spawning interval of 2 days throughout the 120 day monsoon season (June to beginning of October)[17]. Due to numerical

constraints in Vortex v9.99c, n=60 spawning events per year × fecundity of 50 ± 20 eggs per female per spawn, were simulated as n=6 spawning events per year × fecundity of 500 ± 200 eggs per female per spawn. Mating was assumed to be polygynous with a degree of mate monopolisation based on female preference for larger males[20,26]. Male breeding success was assumed to follow a Poisson distribution[19] simulating (without the need for growth data) increased monopolisation by larger males when the proportion of females was reduced. Maturation takes up to 150 days in wild zebrafish[21,23], which is longer than the monsoon breeding season, therefore generations were assumed to be non-overlapping and generation time was assumed to be one year. Given this short generation time, the duration of simulations was limited to 100 years, exceeding the minimum of 40 generations recommended for assessing emergent inbreeding depression vs. purging of deleterious alleles and ultimately population viability[27].

Stochasticity: All models included random environmental stochasticity affecting annual birth, survival rates and sex ratios, as well as demographic stochasticity in vital rates. A 1:100 year catastrophic event (i.e. a dry monsoon) was simulated as part of the sensitivity analysis (Supporting Information: Table 8). Genetic stochasticity was represented by simulating emergent inbreeding depression (see depensatory mechanisms), which is often correlated with genetic drift[19].

Depensatory mechanisms: Depensatory mechanisms, which negatively impact small populations, including inbreeding depression and reduced probability of finding mates[28], were included within the model simulations. Each control model was run with and without simulating chronic inbreeding depression in age 0+ survivorship, which can emerge over future generations in the wild[19]. The level of inbreeding depression simulated was based on 5 lethal equivalent recessive alleles per diploid genome, estimated from juvenile survivorship in our inbred families (Fig. S1) and other wild caught zebrafish[29,30]. We assumed in the PVA model that 50% of the inbreeding depression was due to recessive lethal alleles that were subject to purging, while the remaining 50% was attributed to sub-lethal alleles[31]. We did not simulate increased vulnerability of individuals in small populations to predation or other "Allee" effects[32].

Compensatory mechanisms: Compensatory mechansims, which positively impact small populations include increased survival, fecundity and/or growth when population densities are low. We incorporated density-dependent survival via the Beverton Holt recruitment model[33]. This was applied to age 0+ juvenile zebrafish (up to 32 mm in length), representing a wild outbred population from Mymensingh, Bangladesh, which was studied by Hazlerigg et al.[16] Annual density-dependent survivorship (Equation 1) was entered as a function for age 0+ survivorship in Vortex, assuming the majority of affected fish in the population are 10-20 mm in length. Equation 1 was derived from Equation 2, quantifying daily survivorship[16].

Equation 1: Annual density dependent survivorship = Power (1-(0.99825/(1.00147 * N)); 365)

Equation 2: Daily density dependent survivorship = $e^{cL\wedge d}$ / 1+ (aL + b) * N

Where: a = 0.000133 density dependent mortality constant; b = 0.0028 strength of density dependent mortality at length 10 mm; c = -0.884444 density independent mortality constant; d = -2.7044 strength of density independent mortality exponent; N = abundance (approximates to age 0+ abundance); L = 10 mm in length (majority of age 0+ fish experiencing density dependent mortality/survival).

Table S8

Sensitivity analysis design

Parameter	Inbred population		Outbred population	
	Mean	St Dev	Mean	St Dev
Juvenile survivorship % (age 0+)	4, 5, 6	3, 4	7, 8, 9	3, 4
Adult survivorship % (age 1+, 2+)	5, 10, 19, 30	3, 10	5, 10, 19, 30	3, 10
Fecundity (age 1+, 2+)	20, 30, 40, 50	5, 10, 20, 30, 40	20, 30, 40, 50	5, 10, 20, 30, 40
Carrying capacity K	2000, 3000, 5000, 10000	1000, 2000, 3000	2000, 3000, 5000, 10000	1000, 2000, 3000
Lethal equivalents LE	5, 7, 10		5, 7, 10	
Sex ratio*	18% females : 82% males			

Reference (control) values highlighted in blue.

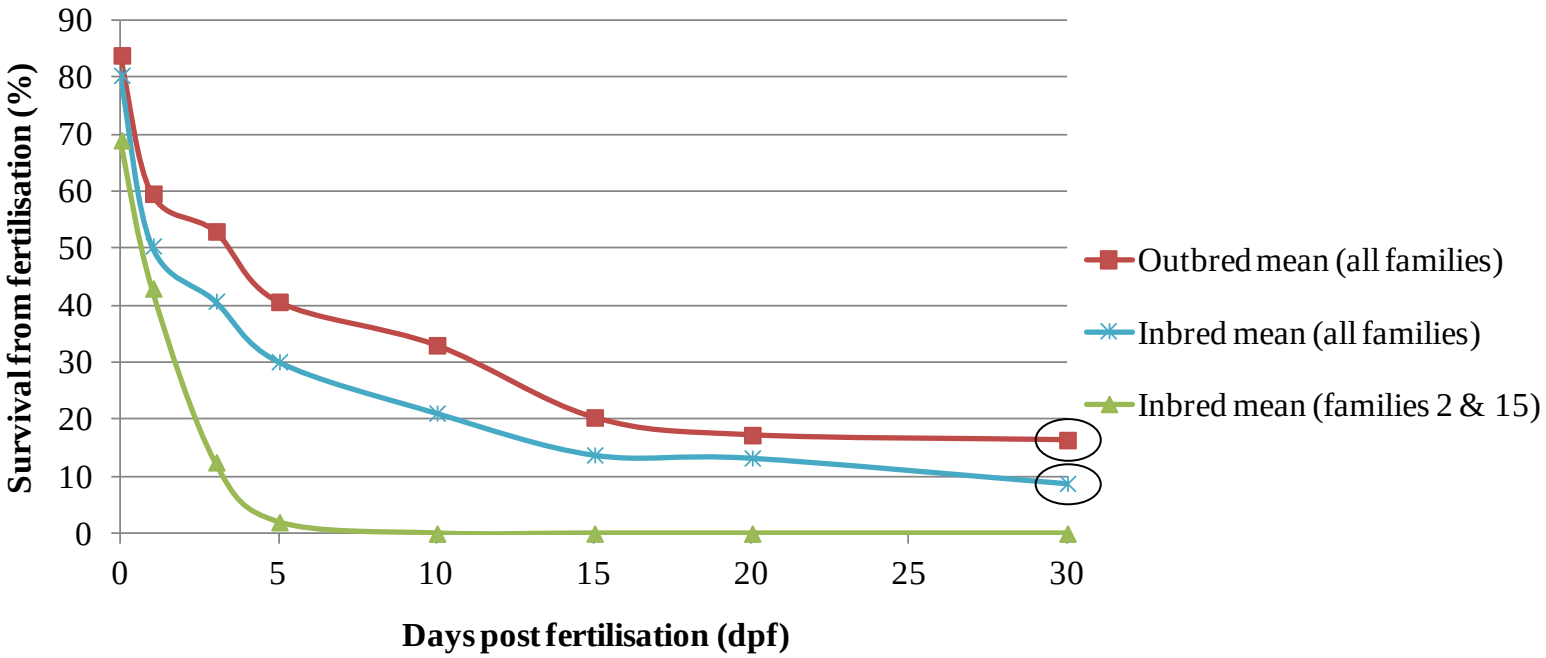
Population sex ratio was fixed initially at 82% males, representing a threshold above which r declined sharply. The remaining demographic rates/input parameters were varied in turn within specified ranges of their mean reference values (Table S8). Scenarios generating significant probabilities of extinction PE>0.05 were re-run on populations with: increased carrying capacity (10000 ± 2000 total individuals); two adult age classes; year-round spawning. Emergent inbreeding depression (based on LE) was generally not included in sensitivity analysis simulations.

Increasing sex ratio skews from critical levels of 80% males to 97% males, shown to impact on zebrafish population growth rate, equated to a 5-fold reduction in female fecundity (Fig. S3.2). Nevertheless, probability of extinction was insensitive to changes in fecundity throughout the range of values reported for zebrafish[16]. These results are indicative of reserve fecundity typical of broadcast spawning fish, r-strategists that produce large numbers of progeny to offset low survivorship[34]. First year survival (age 0+) and inbreeding depression on this vital rate were most influential on limiting population growth and viability (Fig. S3.1), followed by second year survival (age 1+). Zebrafish populations may be restricted to the above two age classes[17], or may also include a third age class (age 2+)[16]. The addition of a third age class (age 2+) was shown to significantly increase population viability, as was increasing carrying capacity (from 2000 to 5000 total individuals, *circa* 60 to 200 adults), indicating the importance of both population structure and size (Fig. S3.3).

A 1:100 year catastrophic event (i.e. an exceptionally dry monsoon) was simulated by adjusting survivorship and fecundity rates to 1/10 of their baseline values for 1 year in each 100 year simulation. This had no perceptible, additional effect on the viability of inbred or outbred zebrafish populations comprised of 2 or 3 age classes.

Figure S1

Survivorship of inbred versus oubred family lines (0-30 dpf)

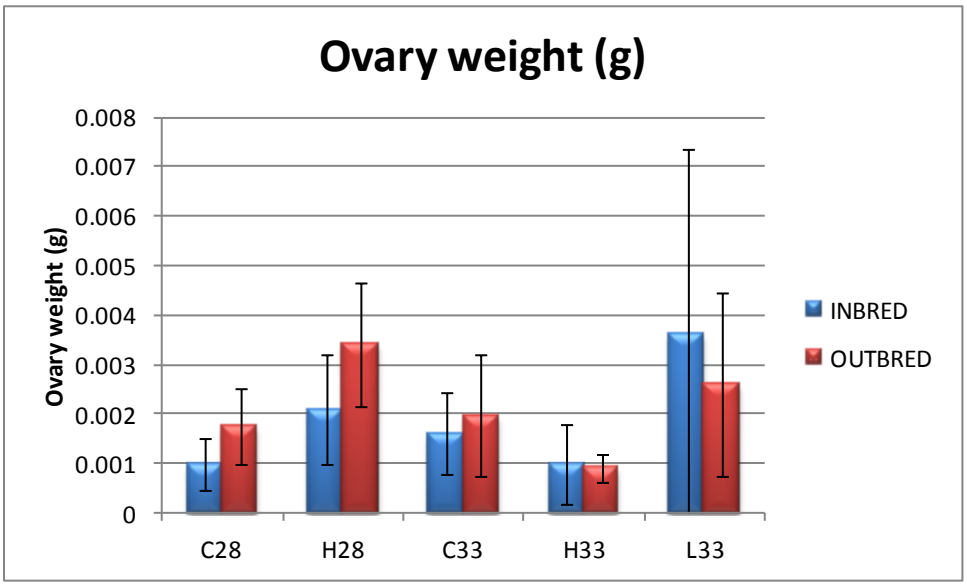


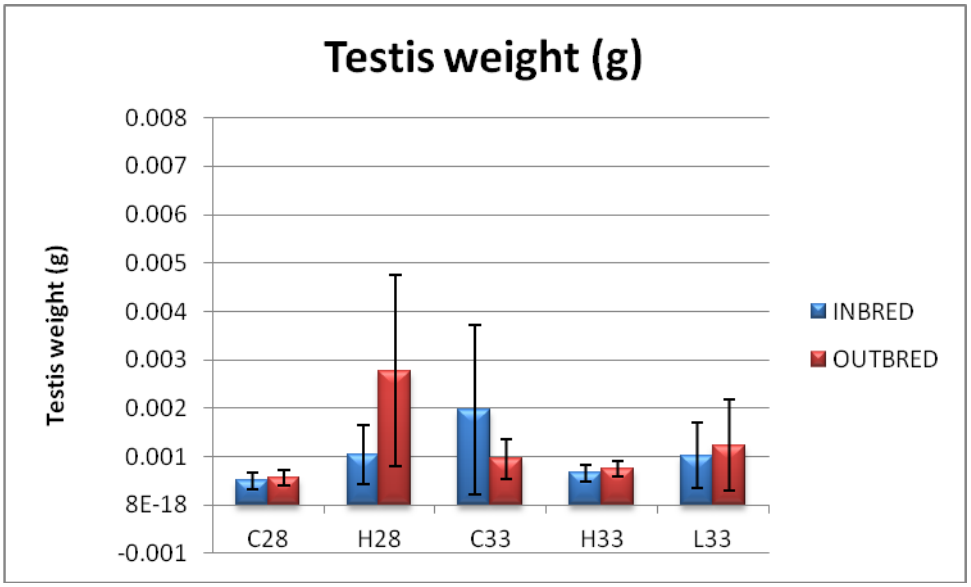
LE = - 2 / *F* * ln (X_I/X_O), where X_I is mean inbred survivorship, X_O is mean outbred survivorship to 30 dpf, *F* is the inbreeding coefficient (here = 0.25) [35]

LE = -2 / 0.25 * ln (8.8/16) = 4.99

Figure S2

Gonad weights at the end of the exposure study (100 dpf)



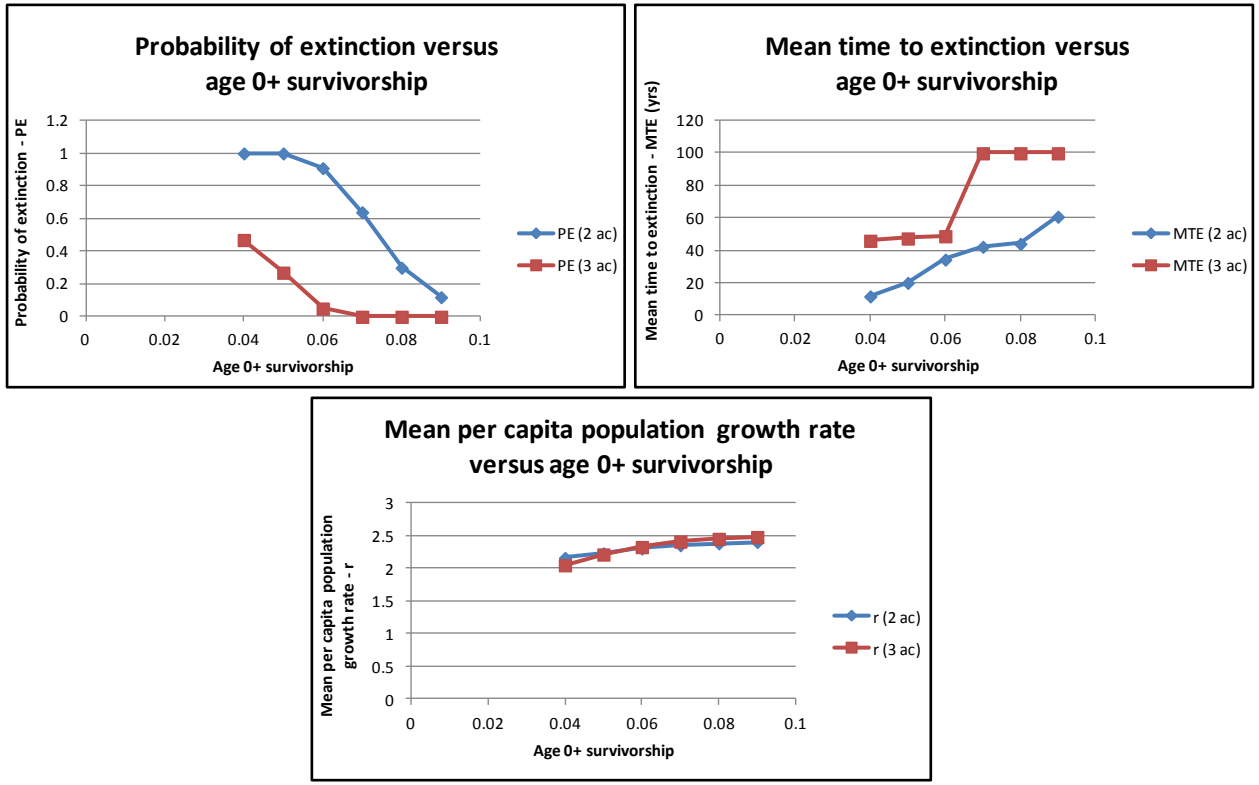


Weights of right side gonads presented (wet weight)

Figure S3

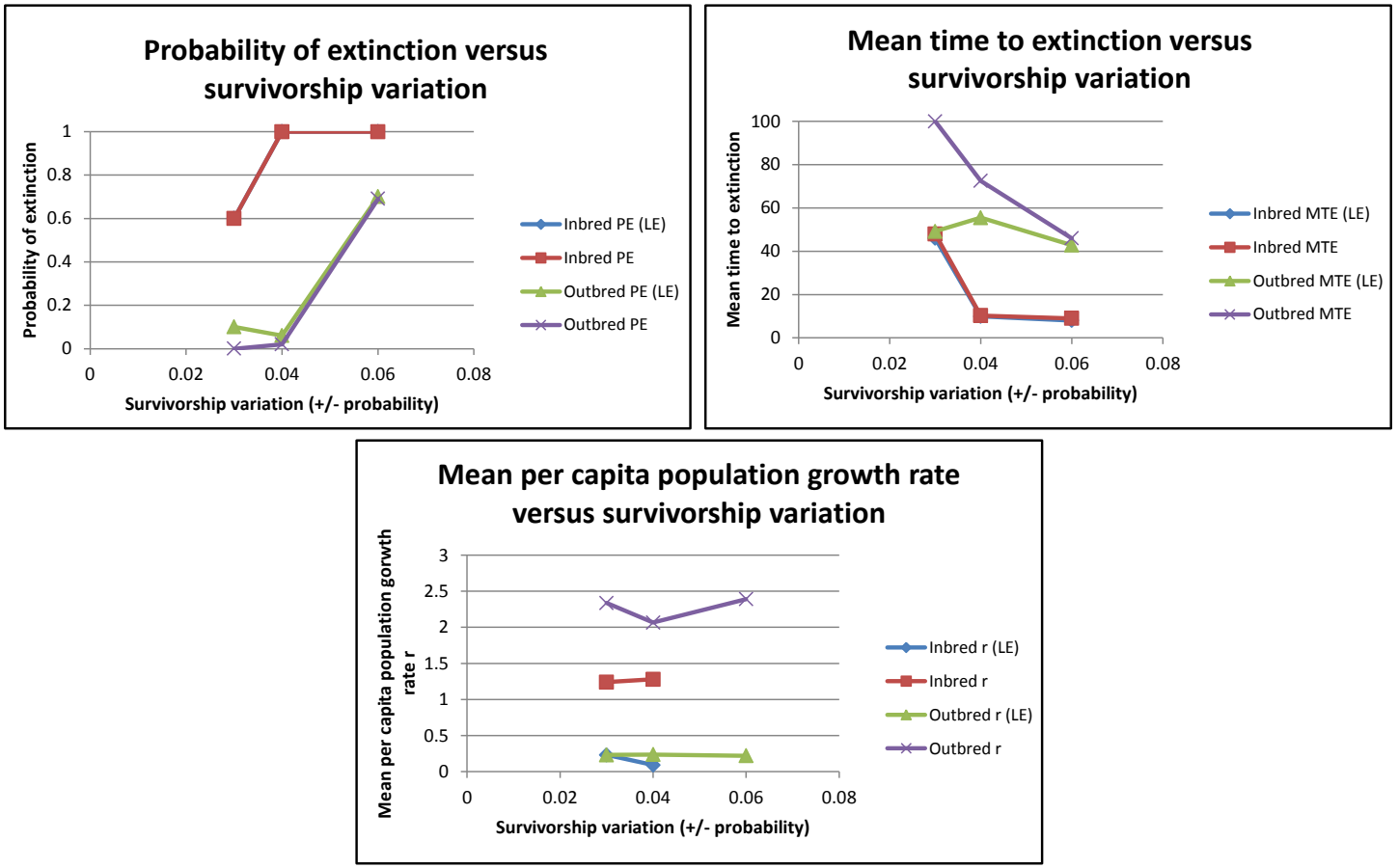
PVA model sensitivity analysis for model input parameters other than sex ratio

Figure S3.1A: Age 0+ survivorship (deterministic change in mean age 0+ survivorship)



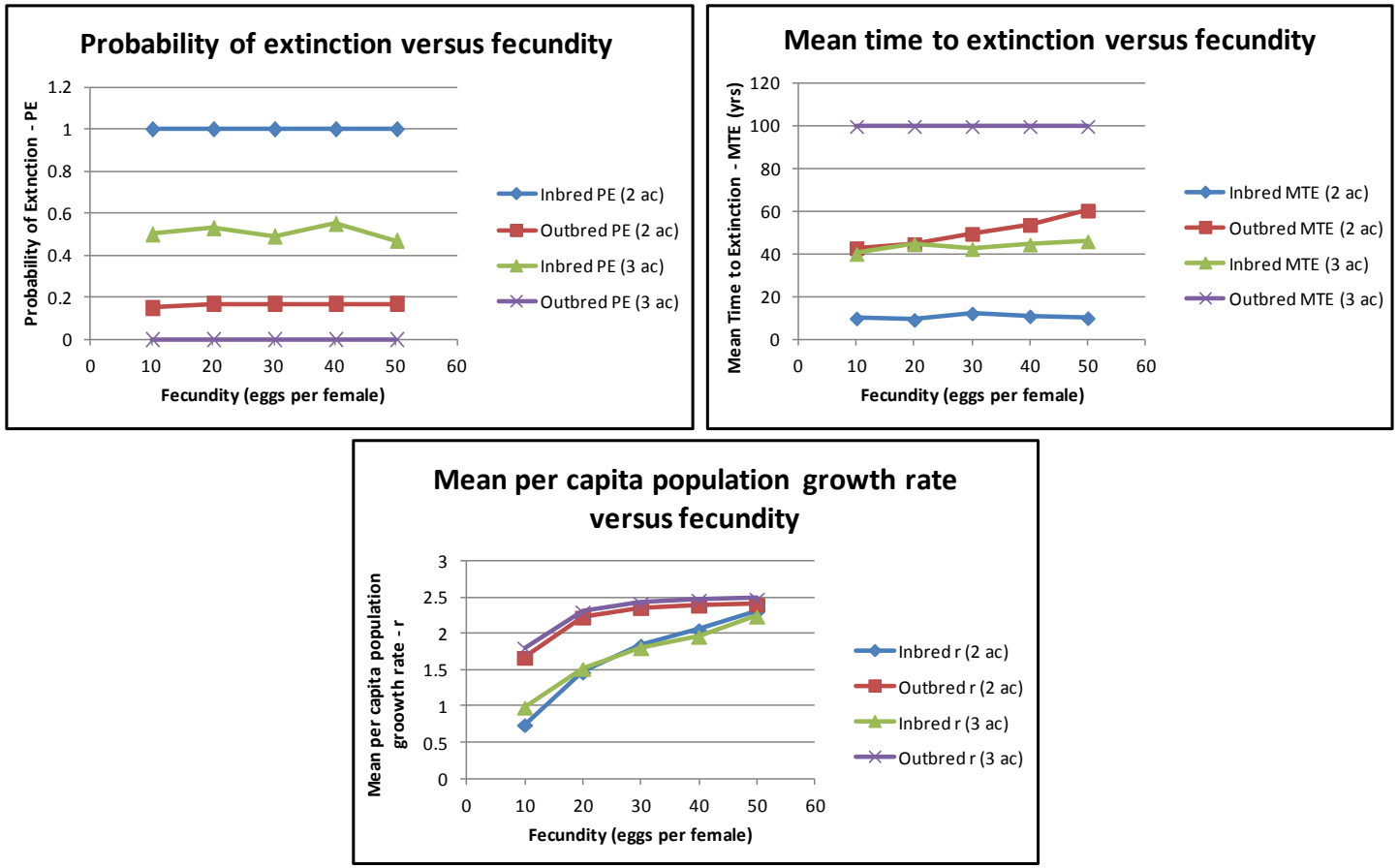
All scenarios simulate breeding on alternate days during the 120 day monsoon spawning season. Simulations in blue represent two age classes (2 ac), in which reproduction is restricted to age 1+ adults. Simulations in red represent three age classes (3 ac), in which reproduction can occur in age 1+ and age 2+ adults. Standard deviation in age 0+ survivorship = ± 3%, total annual carrying capacity = 5000 ± 1000, 18% of population assumed to be female, fecundity = 50 ± 20 viable eggs per female per two days, ≥age 1+ survivorship = 19 ± 3%. Emergent inbreeding depression (based on LE) was not included in deterministic sensitivity analysis simulations. (See Table S8 for other input parameter reference values and ranges).

Figure S3.1B: Age 0+ survivorship variation (change in level of stochastic variation)



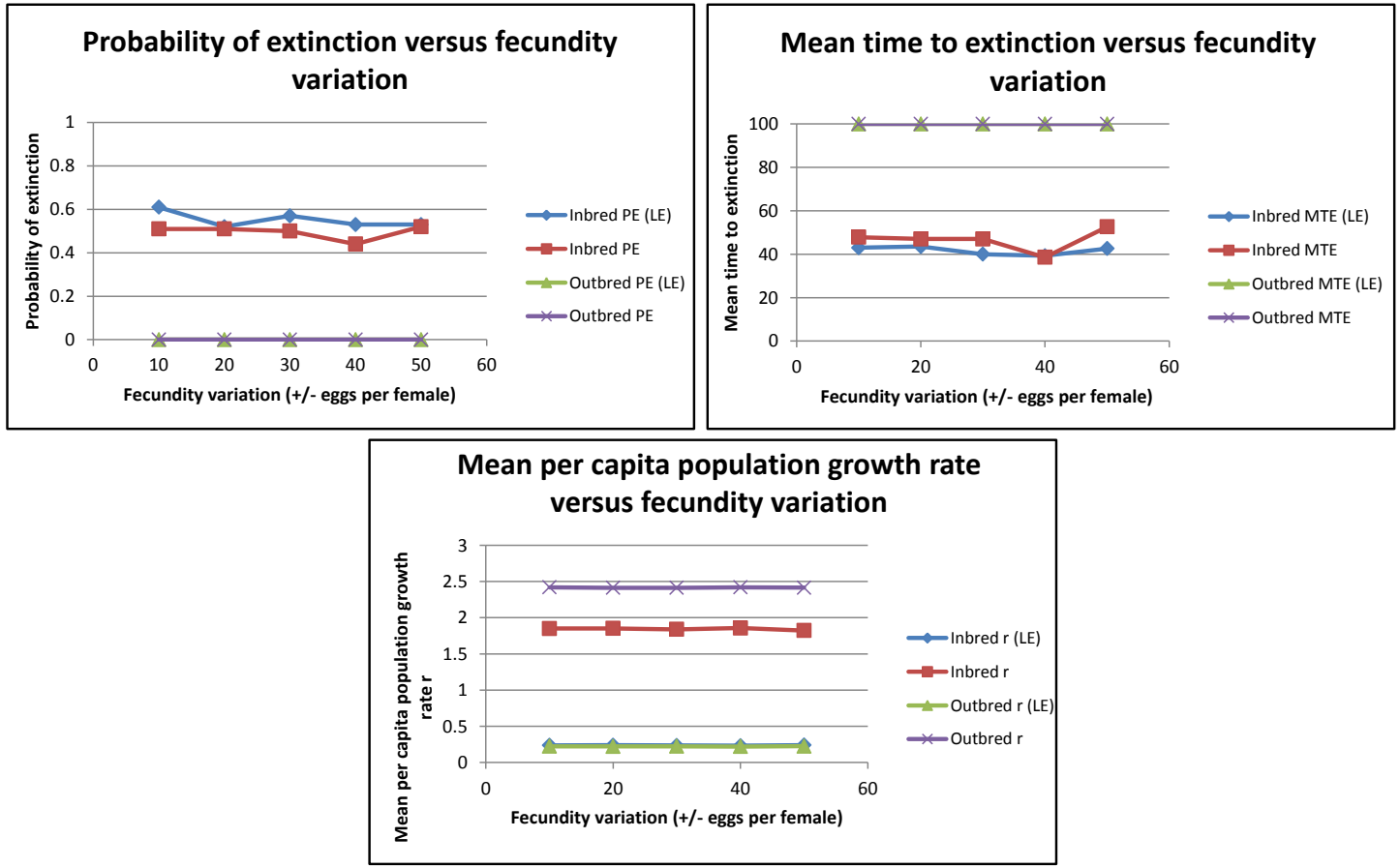
All scenarios were based on the same breeding dynamics as for deterministic sensitivity analysis (Fig. S3.1A), this time for populations composed of three age classes (3 ac). Emergent inbreeding depression was based on 5 lethal equivalent recessive alleles (LE) per diploid genome. Mean age 0+ survivorship = 4% for inbreds or = 9% for outbreds, total annual carrying capacity = 5000 ± 1000, fecundity = 50 ± 20 viable eggs per female per two days, 18% of population assumed to be female, ≥age 1+ survivorship = 19 ± 3%. (See Table S8 for other input parameter reference values and ranges).

Figure S3.2A: Female fecundity (deterministic change in mean female fecundity)



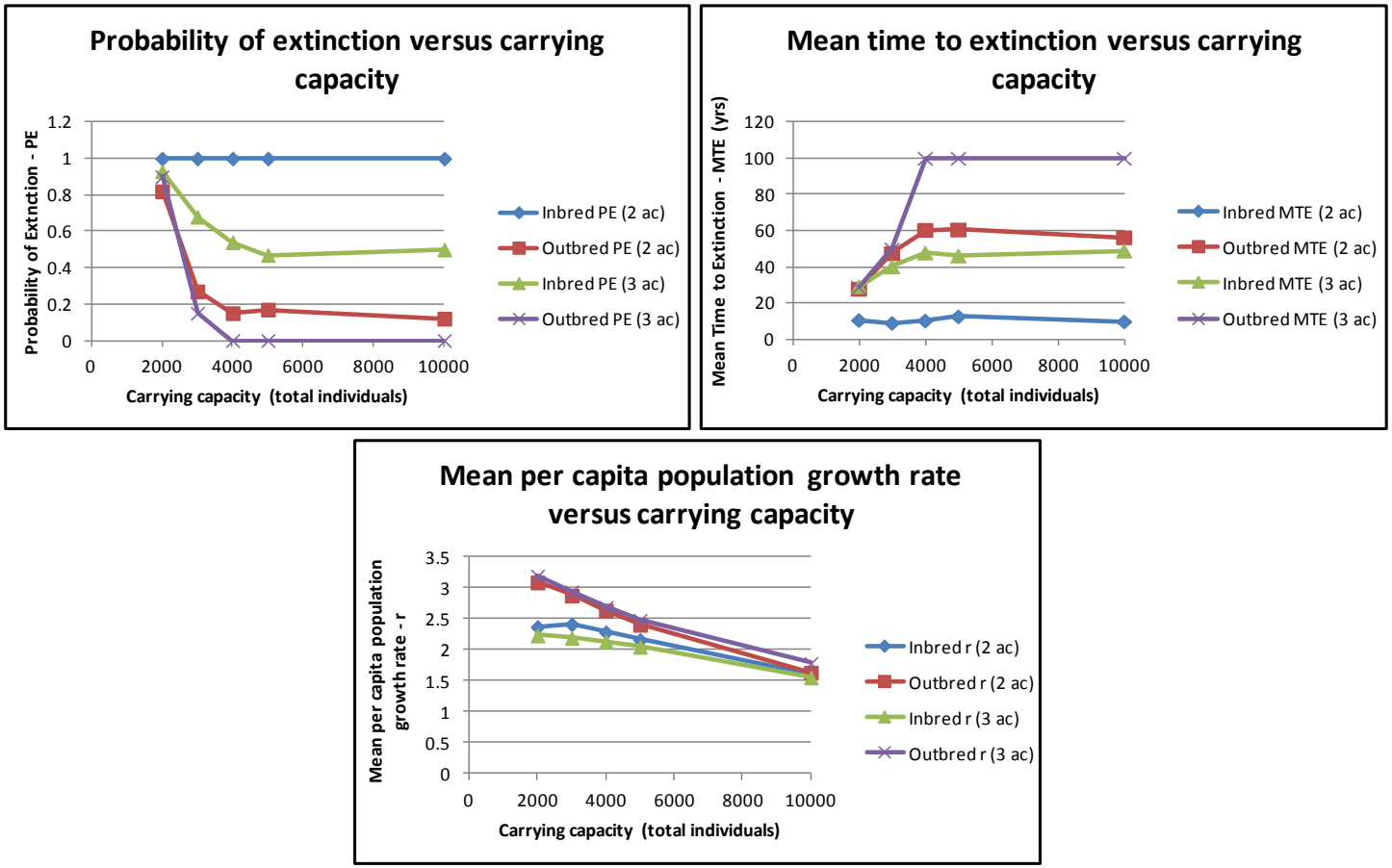
All scenarios simulate breeding on alternate days during the 120 day monsoon spawning season. Inbreds and outbreds were distinguished by age 0+ survivorship (4 ± 3% and 9 ± 3% respectively). Simulations for both inbred and outbred populations were based on two age classes (2 ac), in which reproduction is restricted to age 1+ adults and also three age classes (3 ac), in which reproduction can occur in age 1+ and age 2+ adults. Standard deviation in fecundity = ± 20 eggs per female per two days, 18% of population assumed to be female, total carrying capacity = 5000 ± 1000, ≥age 1+ survivorship = 19 ± 3%. Emergent inbreeding depression (based on LE) was not included in deterministic sensitivity analysis simulations. (See Table S8 for other input parameter reference values and ranges).

Figure S3.2B Female fecundity (change in level of stochastic variation)



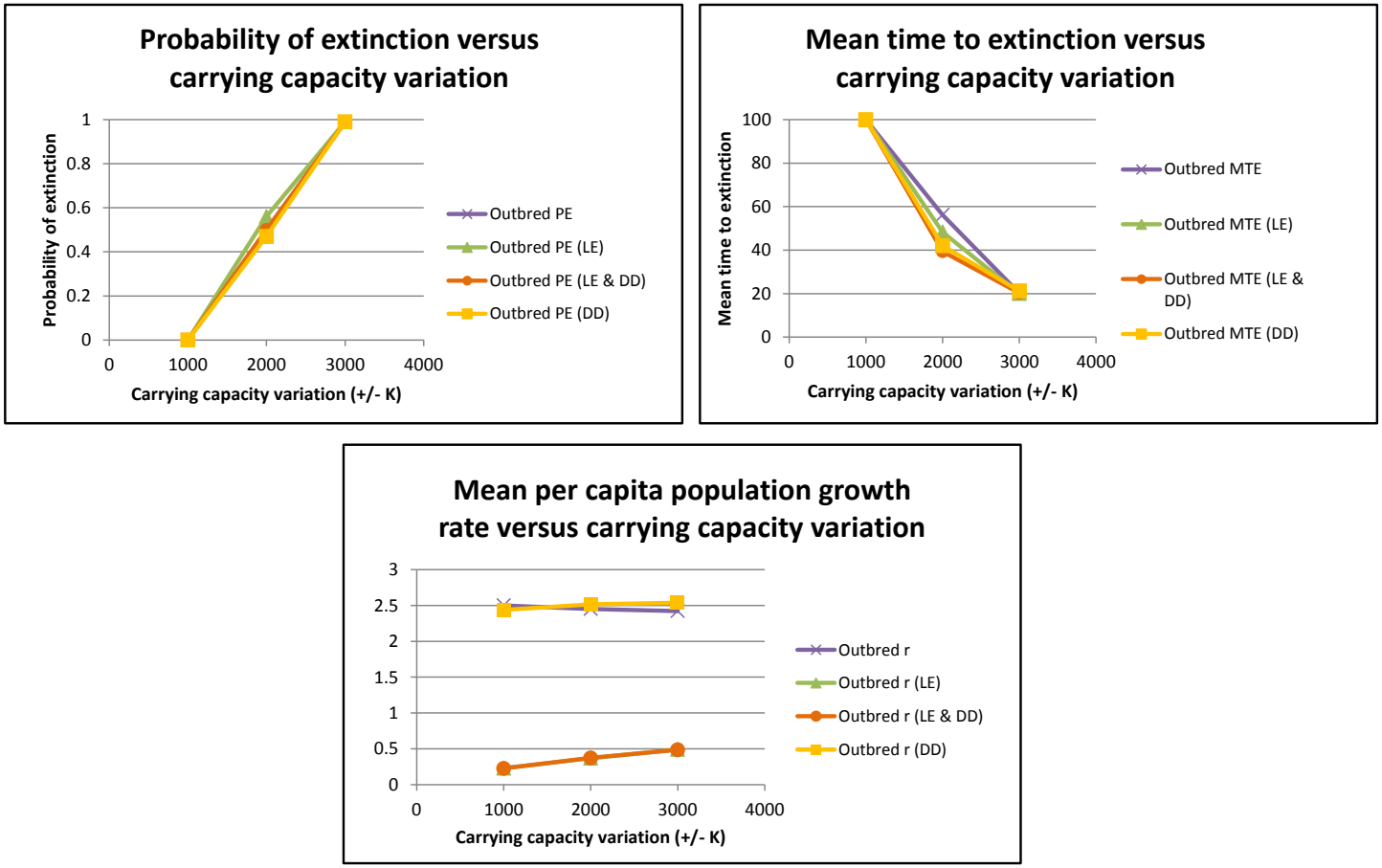
All scenarios were based on the same breeding dynamics as for deterministic sensitivity analysis (Fig. S3.2A), this time for populations composed of three age classes (3 ac). Emergent inbreeding depression was based on 5 lethal equivalent recessive alleles (LE) per diploid genome. Mean fecundity = 50 viable eggs per female per two days, 18% of population assumed to be female, total annual carrying capacity = 5000 ± 1000, ≥age 1+ survivorship = 19 ± 3 (See Table S8 for other input parameter reference values and ranges).

Figure S3.3A: Carrying capacity (deterministic change in mean carrying capacity)



All scenarios simulate breeding on alternate days during the 120 day monsoon spawning season. Inbreds and outbreds were distinguished by age 0+ survivorship (4 ± 3% and 9 ± 3% respectively). Simulations for both inbred and outbred populations were based on two age classes (2 ac), in which reproduction is restricted to age 1+ adults and three age classes (3 ac), in which reproduction can occur in age 1+ and age 2+ adults. Standard deviation in annual carrying capacity = ± 1000, ≥age 1+ survivorship = 19 ± 3%, fecundity = 50 ± 20 viable eggs per female per two days, 18% of population assumed to be female. Emergent inbreeding depression (based on LE) was not included in deterministic sensitivity analysis simulations. (See Table S8 for other input parameter reference values and ranges).

Figure S3.3B Carrying capacity (change in level of stochastic variation)



All scenarios were based on the same breeding dynamics as for deterministic sensitivity analysis (Fig. S3.3A), this time for populations composed of three age classes (3 ac). Mean annual carrying capacity = 5000, fecundity = 50 ± 20 viable eggs per female per two days, 18% of population assumed to be female, outbred age 0+ survivorship = 9 ± 3%, ≥age 1+ survivorship = 19 ± 3%. Density dependence (DD) in age 0+ survivorship was simulated for outbred populations using the Beverton Holt model[33] parameterised according to Hazlerigg et al.[16]. Emergent inbreeding depression was based on 5 lethal equivalent recessive alleles (LE) per diploid genome. (See Table S8 for other input parameter reference values and ranges).

Notes to Figure S3: Sensitivity analysis

Figure S3.1

a) Deterministic variation in mean age 0+ survivorship: A 2.25-fold increase in age 0+ survivorship between inbred and outbred (4 to 9%) fish corresponded with a near 10-fold, largely monotonic reduction in PE (from 1 to 0.12) and a six-fold increase in MTE (from 12 to 61 years).

b) Stochastic variation in age 0+ survivorship: Increasing the standard deviation in age 0+ survivorship from ± 3% to ± 4% had no effect on outbreds with a mean of 9%, but in inbreds, with a mean of 4%, this led to a near 2-fold increase in PE (from 0.6 to 1) and a 5-fold reduction in MTE (from 48 to 10 years).

Figure S3.2

a) Deterministic variation in mean female fecundity: Despite no effect on PE or MTE, a 5-fold reduction in fecundity, from the reference value of 50 ± 20 to 10 ± 10 eggs per female, resulted in a 2.4-fold reduction in mean per capita population growth rate in inbreds (from r = 2.4 to <1) and a 1.33-fold reduction in outbreds (from r = 2.4 to <1.8). This change in r was comparable with that caused by reducing the proportion of females to from 18% to 2.8% (Figure 5 in main manuscript).

b) Stochastic variation in female fecundity: Altering the standard deviation in fecundity from ±10 to ±40 about a mean of 50 eggs per female, per every two days had no effect on any of the PVA outputs.

Figure S3.3

a) Deterministic variation in mean annual carrying capacity: A 2.5-fold increase in annual carrying capacity (from 2000 ± 1000 to 5000 ± 1000 total individuals) led to a 5-fold decrease in PE (from 0.82 to 0.17) and a 2-fold increase in MTE (from 28 to 60 yrs) in outbred populations with age 0+ survivorship = 9%, while there was no effect on inbred populations with age 0+ survivorship = 4% and PE = 1 (unchanged). A further 2-fold increase in environmental carrying capacity from the reference value of 5000 ± 1000 to 10000 ± 2000 total individuals had no effect on PE and MTE on either population.

b) Stochastic variation in annual carrying capacity: A 3-fold increase in standard deviation in annual carrying capacity (from ±1000 to ±3000 total individuals about a mean of 5000 individuals) led to an increase in PE from 0 to 1 and a reduction in MTE from >100 to 20 years for outbred populations with and without emergent inbreeding depression (LE) and/or density dependent compensation in age 0+ survivorship.

Figures S3.1-S3.3

Increasing age1+ survivorship from 0 to 50%, allowing 50% of age 1+ zebrafish to survive and breed successively in their third year (age 2+), and creating three age classes (3ac) rather than two (2ac), reduced PE substantially in outbred populations (PE_{3ac} = 0; PE_{2ac} = 0.18) and inbred populations (PE_{3ac} = 0.43; PE_{2ac} = 1).

Additional notes

There was also no noticeable population effect from simulating year-round spawning, as opposed to seasonal monsoon spawning. Varying age 1+ survivorship, fecundity and LE within ranges reported for zebrafish (Table S8) also had no effect on PE or MTE.

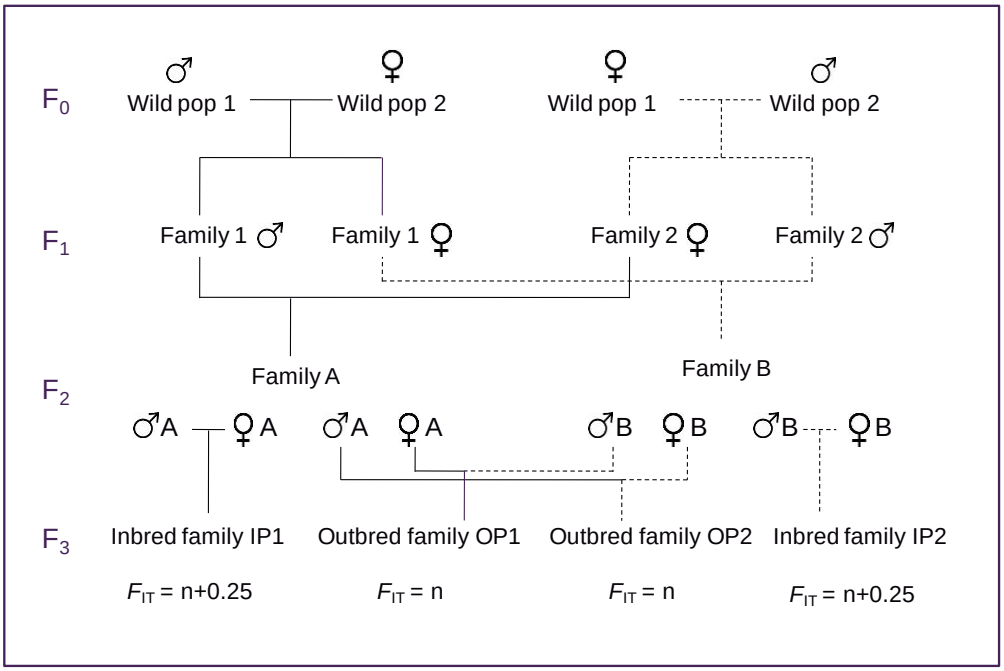
Simulating inbreeding depression based on 5 LE may represent a significant underestimate, since the impact of inbreeding has been shown to be more than three times greater when fitness components other than juvenile survival are taken in account over the full life-cycle, including adult survival, fecundity and mating success[36]. Furthermore, inbreeding depression may be up to seven times greater in the wild compared to that found in laboratory maintained populations[37]. Consequently we performed additional simulations based on more conservative values of 15 and 35 LE, but the results ($r = 0.17 - 0.19$) were not significantly different to 5 LE ($r = 0.19$).

Simulating density dependent compensation in age 0+ survivorship, using the Beverton Holt model[33], parameterised for outbred zebrafish[16], had no effect on PVA model outputs except for maintaining per capita population growth rate (r) when population sex ratio was skewed to >97% males (<3% females) (see Figure 5 in main manuscript).

Simulating a 1:100 year catastrophic event (e.g. an exceptionally dry monsoon, which reduced mean survivorship and fecundity to 10% of their baseline values for one year in a 100 year simulation) had minimal effect on populations with three age classes and 18% females: this increased PE by 0.1.

Figure S4

Pair breeding design for generation of inbred and outbred lines (F_3 generation) of zebrafish.



The notation "n" in the inbreeding coefficients of the F_3 generation reflects their unknown pedigree, relating to their wild great grandfathers and grandmothers (F_0 generation). Two of the 20 inbred F_3 family lines failed to recruit. F_1 families 3-24 and corresponding F_2 families C-T and F_3 families IP/OP 3-20 (from Table S2) not depicted.

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