Electronic Supplementary Material

Mating portfolios: bet-hedging, sexual selection and female multiple mating

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1. Supplementary material and methods

Reproductively mature individuals were collected from two locally available (~8km apart) populations in Fremantle (32° 3'35.76"S 115°44'1.30"E) and Woodman Point (32° 8'5.06"S 115°44'18.05"E) in Western Australia. Animals were housed in recirculating seawater tanks at the University of Western Australia until used for the fertilizations (within 5 days of collection). Trials took place during the peak of the 2009 and 2010 breeding seasons (end of March-early April 2009, and early March-end of May 2010). For Experiment 1, seven blocks were run in 2009 and five in 2010; all blocks in Experiment 2 were run in 2010. Each individual was used just once in the experiments.

Experiment 1: Bet-hedging in the absence of sexually selected processes

Immediately prior to the fertilization trials, adults were placed in individual containers with clean seawater (to prevent cross-contamination) and induced to spawn with a intracoelomic injection of 3% KCl [1]. Female gametes were collected and diluted so that the final concentration was standardized at 50 eggs/ml in a volume of 200 ml filtered seawater (FSW). The gametes from each male were adjusted, using an improved Neubauer haemocytometer (three replicate counts per male), so that the final concentration was standardized at 14 x 10^5 sperm/ml in 50 ml of FSW. Fertilizations were performed in Petri dishes (5.5 cm diameter) with 10 mL of the egg solution and 10 mL of the sperm solution. Thus, each batch of female eggs contained approximately 500 eggs and the ratio of sperm to eggs was kept constant for all fertilizations within and among blocks at 28000 sperm per egg. This concentration resulted in fertilization rates averaging 38% (see results in main text). All fertilizations within each block took place within 30 min. of gamete collection. Gametes were mixed in the Petri dishes at a temperature of 22°C and the resulting solution was kept under aeration until fertilization was assessed.

Each of the two embryo vessels (see text) from each mating event and treatment type was assigned haphazardly to one of two environmental conditions: Environment A (pH 8) and Environment B (ph 7; acidic environment). Several studies carried out in *H. erythrogramma* have found weak or non-significant effects of acidic pH conditions in the range from 7.5-7.6 to 8.1 on early post-zygotic development [2, 3]. In Environment B we imposed conditions that were more acidic than pH 7.5 and to avoid potential ceiling effects regarding mortality rates

the pH of the Environment B was not maintained after embryo transfer; the purpose of Environment B was to impose a sudden but short-term drop in pH levels, mimicking a punctual change in local environmental conditions due to (for instance) the presence of pollutants in the water. Thus, the differences in pH between the two environments were expected to be larger during the first hours of embryo development and become gradually smaller over time. Measurements taken 24 h after embryo transfer confirmed that at this time pH of both environments converged at pH 8. The containers housing the developing embryos were fitted with lids to prevent evaporation and had a constant supply of air until measures of offspring viability were taken 8 d after fertilization. All samples were kept at 22°C.

Offspring viability was measured by counting the number of offspring that were alive in the containers 8 days after fertilization. Fertilized eggs start turning into free-swimming larvae at approximately 15-h post-fertilization, when the gastrula emerges from the fertilization membrane and jelly coat, and after 40 h the larvae can commence metamorphosis into the juvenile stage [4]. Typically, our samples at age 8 days consisted of a combination of larvae and juveniles. Each offspring was individually inspected under a Leica MZ7.5 stereomiscrocope. An individual was classified as alive if it was moving on its own, if it moved after gently touching it with a probe, or whether its cilia were moving.

Summary of experimental design features

1. Our experiment enables a comparison of fitness returns from two mating strategies (monandry or polyandry) controlling for variance due to female identity (i.e., the genetic background of the individuals following either strategy is kept constant). The modified maternal full sib-half sib design controls for confounding effects including female ageing, maternal effects (e.g., differential maternal investment among offspring from different treatments), and uncontrolled stochastic environmental variance, which would otherwise preclude a clean examination of the consequences of mating strategy on female fitness estimated across generations.
2. The three mating events per treatment simulate different reproductive bouts whereby each mating event can be taken as one generation in which a given female follows a given mating strategy (monandry or polyandry). The approach therefore has the properties of a transgenerational study allowing us to investigate what would happen if we could ever

measure in nature clonal lineages following both mating strategies across generations, without the constraints and limitation mentioned in the preceding point.

3. Sampling error affecting our estimation of fitness variance is minimized because sample sizes are standardized across replicates.

4. In Experiment 1 eggs in each Petri dish of the polyandrous treatment are fertilized by the sperm of a different male before combining the same number of zygotes of each of the three males into a single vessel. That is, paternity-biasing mechanisms (sperm competition and sperm selection) [1, 5-7] are absent (see Experiment 2 for a variation of this and further information and see also [8, 9]). Thus, bet-hedging can be assessed without the confounding effects of male, female or male-by-female driven paternity biases, something that is generally unattainable in most studies. Obviously, because of the model system and protocol used there was not any room for potential pre-copulatory based biases either (e.g., female choice) to influence the results, which complies with the bet-hedging assumption that the females' ability to discriminate male quality before mating is unreliable or non-existent [10].

5. The design allows the examination of genetic benefits (offspring viability) arising from bet-hedging mechanisms, but also bet-hedging effects for direct benefits (fertilization rates; see text).

Experiment 2. Bet-hedging plus sexually selected processes

Experiment 2 is identical to Experiment 1 apart from the fact that the sperm from the three males in each mating event within the polyandrous treatment were mixed prior to fertilization instead of being placed unmixed into separate Petri dishes. For each mating event three fertilization trials were run in which 10 ml of the mixed sperm solution were added to 10 ml of egg solution. As with Experiment 1, 12 blocks were carried out and fertilization rates were calculated using 100 eggs per fertilization dish (n dishes = 213).

Data analysis with Monte Carlos simulations.

i) Test of the genetic bet-hedging hypothesis in Experiment 1. The establishment of the three "mating events" in our set up was designed to simulate different generations but the labelling of these three generations is arbitrary. That is, there is no reason to calculate W_{WG} for, say, generation *x*, using only the scores in what we called "mating event 1" since our labelling of mating events implies no temporal variation *per se*. For this reason, our analyses employ a

resampling protocol where the order of the generations is shuffled within block before calculating W_{BG}. Note, however, that one male is shared between the scores of the two mating strategies within each block and mating event (see Figure 1a); the resampling protocol accounts for this and so the shuffling of generations is carried out within each block whilst maintaining the paired structure of the data. We calculated the statistic of interest, Poly W_{BG} - Mono W_{BG}, for each of 10,000 iterations of the resampling protocol so that a distribution of this statistic is obtained. The mean and confidence limits (CL) from this distribution were then inspected to assess the effect size of the advantage (or disadvantage) of polyandry via bet-hedging. Resampling and Monte Carlo simulations were carried using the PopTools (3.1.1) add-on in Excel [11].

ii) Test of the genetic diversity bet-hedging hypothesis in Experiment 1. *Fluctuating environments (ABA or BAB) analyses*: The scores used to calculate W_{WG} are, as in the section i) above, the proportion of surviving offspring for each female and generation, but instead of using the data from a single environment the resampling feeds on the data from the different environments. Apart from this additional resampling step, the implementation of the resampling protocol was unchanged (i.e., generations are shuffled within block and the paired structure of data belonging to different mating strategies within block and generation is maintained). *Averaging across environments A+B analysis*: In this analysis W_{WG} is calculated using the average (arithmetic mean) of the proportions of offspring alive in environments A and B for each female and generation. Apart from this difference, the resampling protocol is similar to the one described above to test the genetic bet-hedging hypothesis (i).

iii) Test of the direct benefits bet-hedging hypothesis in Experiment 1. We investigated the scope for polyandry to generate direct benefits through bet-hedging by looking at differences in fertilization rates between mating strategies. In this case, for each female we have data from three fertilization events (one per Petri dish) for each generation and strategy. The scores used per block, generation, and strategy to calculate W_{WG} were thus the arithmetic mean of the fertilization rates obtained in these three fertilization events. We only imposed environmental variation at the level of zygote survival and thus the environmental factor has no bearing on fertilization rates. Fertilization rates were always calculated on the basis of 100 eggs per fertilization dish (n fertilization dishes = 216). The direct benefit of polyandry was calculated

using the analysis and resampling protocol described in section (i) above, but using fertilization rates instead of offspring survival scores.

iv) Experiment 2. The test of the genetic bet-hedging hypothesis, test of the genetic diversity bet-hedging hypothesis, and the test of the direct benefits bet-hedging hypothesis, was carried out with analyses and resampling protocols that are identical to those described for Experiment 1 in sections i), ii) and iii), respectively.

2. Supplementary Results: Male and female effects on fertilization success.

The direct benefits of polyandry through bet-hedging mechanisms and/or sexually selected processes are contingent on male effects or male-female interactions determining fertilization. Our data from the monandrous treatment can be used to estimate male and female effects on fertilization rates. These data, for each experiment, consist of a series of females (n females = 12) paired each with three different males (n males = 36), and three fertilization replicates for each male-female combination (n replicates = 108) (see Figure1a in main text). Male and female effects on fertilization rates (proportion of eggs fertilized) were investigated with generalized linear mixed-effects models with binomial error structure and logit-link function that were run using the glmer function of the lme4 package [12] in R 3.0.0 [13]. Female identity and male identity (nested within females) were entered as random effects. To account for overdispersion an observation-level random effect was also included in the model [14]. The command cbind was used to compose the response variable as a binomial vector with the number of fertilized and unfertilized eggs in each replicate. Standard errors around the estimates of variance components were calculated by jackknifing across female families [15, 16]. Significance of the random effects was assessed using L:R tests [17, 18]. We found consistent male effects on fertilization rates in both experiments (Supplementary Table 1).

3. Supplementary Table S1.

Table S1. Male and female effects on fertilization rates in both experiments.

	N_{eggs}	N_{rep}	*Mean proportion fertilization rates* ± SE	N_{fem}	N_{male}	V_{fem}	V_{male}	P_{fem}	P_{male}
Experiment 1	10800	108	0.43 ± 0.03	12	36	1.50 ± 1.02	3.67 ± 0.98	0.106	<<0.001
Experiment 2	10800	108	0.57 ± 0.03	12	36	2.67 ± 1.64	3.10 ± 1.06	0.009	<<0.001

The table shows total number of eggs scored (N_{eggs}), number of replicates (N_{rep}), the mean proportion of eggs fertilized across replicates and associated standard error, number of females and males (N_{fem}, N_{male}), variance components for female and male effects (V_{fem}, V_{male}) and their standard errors calculated with jackknifing, and the probability associated to L:R tests for female and male effects (P_{fem}, P_{male}). We refrain from interpreting female effects in the text because they are likely inflated by maternal effects.

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