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

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Polyandry (female multiple mating) has profound evolutionary and ecological implications. Despite considerable work devoted to understanding why females mate multiply, we currently lack convincing empirical evidence to explain the adaptive value of polyandry. Here, we provide a direct test of the controversial idea that bet-hedging functions as a risk-spreading strategy that yields multi-generational fitness benefits to polyandrous females. Unfortunately, testing this hypothesis is far from trivial, and the empirical comparison of the across-generations fitness payoffs of a polyandrous (bet hedger) versus a monandrous (non-bet hedger) strategy has never been accomplished because of numerous experimental constraints presented by most 'model' species. In this study, we take advantage of the extraordinary tractability and versatility of a marine broadcast spawning invertebrate to overcome these challenges. We are able to simulate multi-generational (geometric mean) fitness among individual females assigned simultaneously to a polyandrous and monandrous mating strategy. Our approaches, which separate and account for the effects of sexual selection and pure bet-hedging scenarios, reveal that bet-hedging, in addition to sexual selection, can enhance evolutionary fitness in multiply mated females. In addition to offering a tractable experimental approach for addressing bet-hedging theory, our study provides key insights into the evolutionary ecology of sexual interactions.

1. Introduction

Nearly three centuries ago, Daniel Bernoulli wrote a paper on choice decisions under risk that has greatly influenced economic theory and ecological and evolutionary thinking [1,2]. In that work, Bernoulli emphasized the importance of the concept 'utility' and sketched the basic principles governing successful choice behaviour under uncertainty. A suggestion derived from Bernoulli's calculations is that 'it is advisable to divide goods which are exposed to some danger into several portions rather than to risk them all together' (p. 30, 1954). This risk-spreading strategy applies not only to goods or economic transactions, but also to evolutionary fitness. The analogy of not *putting all of ones eggs in one basket* can succeed as a strategy because it protects against complete failure, i.e. null fitness, even if it implies that the prospects of high returns are sacrificed [3–5]. Gillespie [6,7] formalized this *bet-hedging* theory within an evolutionary framework and showed that selection can work to reduce variance in fitness at the expense of average fitness, and that the strength of this selection decreases as population size increases (see also [8]). Bet-hedging, together with adaptive tracking and phenotypic plasticity, are evolutionary responses that facilitate persistence in the face of environmental fluctuation [9].

Bet-hedging, in the form of a 'conservative strategy' (e.g. producing a generalist phenotype) or a 'diversified strategy' (i.e. producing a diverse array of phenotypes) is a strategy that is *neither optimal nor a failure across all environments (sensu [9])*. Selection against demographic stochasticity favouring reductions in variance

rather than a maximization of the mean (i.e. bet-hedging) has been invoked in the evolution of numerous life-history traits, including offspring size, offspring numbers, hatching synchrony, diapause, seed dormancy, timing of germination, timing of flowering, sex-biased dispersal, etc. [4,7–15]. Remarkably, however, there is an alarming paucity of robust empirical tests of bet-hedging theory [9,16], and this is especially applicable to the evolution of polyandry—where females mate with multiple males during a single reproductive episode.

Polyandry has important evolutionary and ecological implications because it extends the opportunities for sexual selection (female choice and male–male competition) beyond mating. By facilitating post-copulatory sexual selection, polyandry generates intense selection for male and female adaptations that allow individuals to gain control over paternity biases [17]. Despite considerable theoretical and empirical attention given to polyandry, including its consequences for population extinction and conservation [18–21], understanding why females mate multiply in the face of the well-established direct (and indirect; [22]) costs of mating remains a major challenge. Most studies that consider this problem test whether females obtain benefits from mating multiply, either directly by increasing reproductive success [23] or indirectly, for example by promoting post-copulatory sexual selection (i.e. sperm competition or cryptic female choice) that ultimately favours high-quality/compatible males when sperm compete to fertilize eggs [24–28]. By contrast, female mating rate variation can be non-adaptive if it results from indirect selection on males due to intersexual genetic correlation in mating behaviour [29], or from convenience polyandry due to interlocus sexual conflict [30].

An additional adaptive explanation, mainly confined to theory, is that polyandry functions as a bet-hedging strategy. Bet-hedging has the potential to play a key role in the evolution of polyandry by bringing immediate benefits to females that mate with multiple males. For example, a mutation that increases a female's propensity to mate multiply may lead to the acquisition of a direct or genetic benefit simply because it can spread risk. Females mating indiscriminately with several males would have the potential to enjoy this bet-hedging-derived benefit, whereby multiple mating would protect against the risk of failed reproduction (zero fitness) arising from male infertility problems, or low genetic quality and/or genetic compatibility leading to non-viable offspring. Such bet-hedging benefits to multiply mated females, either direct or genetic, do not need to invoke mate selection (including sperm selection) or male–male competition (including sperm competition), just multiple mating.

Polyandry as a way of facilitating bet-hedging represents a strategy that deals with 'sampling errors' arising from non-existent or inaccurate information on male quality or with the unpredictability of the future environmental conditions that the offspring will experience. The possibility that polyandrous females garner genetic benefits in the form of increases in the genetic quality of the offspring through bet-hedging was first suggested by Watson [31], and investigated theoretically by Yasui [26,32]. Several subsequent studies have examined the potential for multiple mating to function as a bet-hedging strategy, or addressed related topics such as the possibility that increases in within-clutch genetic diversity generate benefits for multiply mated females [33–36]. To the best of our knowledge, however, no study has compared *geometric mean fitness of monandrous and polyandrous females across generations*, which is

key to testing bet-hedging theory. The concept of geometric mean fitness (the n th root of the product of n within-generation fitness scores) is ideal for measuring intergenerational fitness in the face of stochastic environmental fluctuation [10,12], because it accounts for the multiplicative nature of fitness over generations [2,4,16]. If fitness for a given genotype is zero in generation z (i.e. goes extinct in that generation), then the fitness of that genotype across generations x, y, z is not the arithmetic mean of the fitness of these three generations, but zero. Importantly, the geometric mean fitness has the key property of accounting for the risk inherent in having high variance in fitness [3,4,8].

At least three bet-hedging hypotheses have been proposed to explain polyandry. First, the *genetic bet-hedging hypothesis* addresses fitness variation within a stable environment and suggests that polyandrous females reduce the risk of having all their offspring sired by a male of poor genetic quality [26,36]. The offspring from monogamous females are thus expected to exhibit either very high or very low fitness, depending on whether the female mates (just by chance) with a suitable or unsuitable male, while the offspring of polyandrous females is expected to exhibit on average intermediate viability. Hence polyandrous females would exhibit reduced variance in fitness; they would sacrifice the prospects of very high fitness but would reduce the risk of losing entire clutches because of low offspring viability. Second, the *genetic diversity bet-hedging hypothesis* examines variation in fitness in fluctuating or unpredictable environments and suggests that due to the diversity of paternal genotypes in the offspring of a polyandrous female, the likelihood that at least some offspring will survive in a changing environment is higher than when offspring genotypes are less diverse [26]. Finally, in some situations bet-hedging could also result in direct benefits (*direct benefits bet-hedging hypothesis*), for example as insurance against infertility (see Discussion).

Despite considerable progress in developing the theoretical framework surrounding bet-hedging theory, empirical support for these ideas is largely lacking or incomplete [9]. A likely explanation for this is that testing bet-hedging theory is empirically challenging. This is because an ideal test of bet-hedging theory would compare the fitness of the two strategies, namely monandry and polyandry in the case of female multiple mating, across successive generations while holding female identity constant so that any fitness differences can be attributed exclusively to mating behaviour rather than other factors. Clearly, such tests are impractical for most species, where assigning individual females to both mating strategies (monandry and polyandry) across multiple generations would be impossible or encompass unacceptable compromises. For example, such a design might entail staging matings across successive reproductive bouts, which in turn would entail confounding factors, including ageing effects, mating history effects, loss of condition, depletion of resources allocated to reproduction or ecological variance influencing the outcome of each reproductive bout. Even without such constraints, maternal effects may confound the assessment of fitness pay-offs of the two mating strategies, because females may allocate more resources to offspring when they mate multiply, or invest differentially in offspring according to offspring parentage. Finally, genetic benefits attributable to bet-hedging are difficult to disentangle from those due to sexual selection; polyandry typically results in sexually selected processes that bias paternity towards certain sires [25].

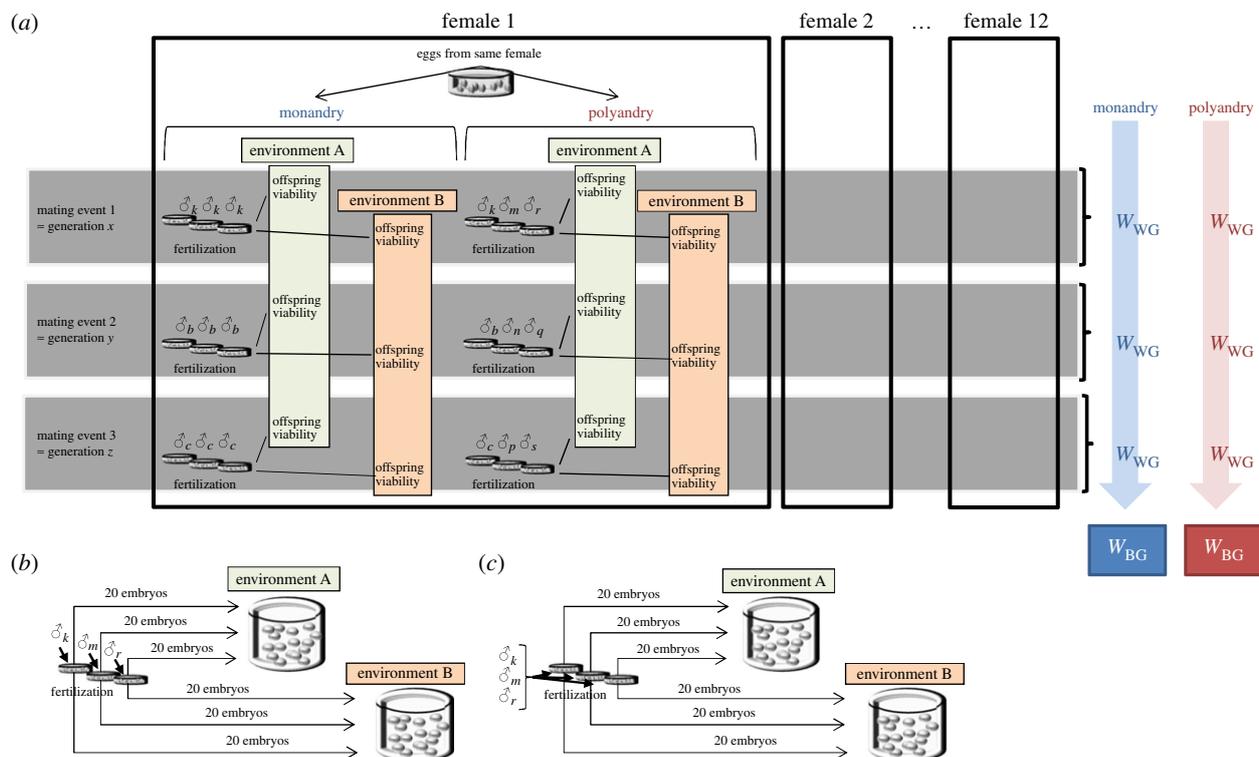


Figure 1. Outline of the experimental design. (a) Within-generation fitness (W_{WG} ; arithmetic mean) for each treatment (mating strategy: monandry or polyandry) and environment can be calculated using the offspring viability scores across females for any given simulated generation. The three W_{WG} values corresponding to the three generations per mating strategy are then used to calculate between-generations fitness (W_{BG} ; geometric mean). (b) In Experiment 1, gametes from the three males in any given simulated generation within the polyandrous treatment were not mixed; the sperm of only one male was used in each fertilization dish. From each of these three fertilization dishes per generation, 20 zygotes were randomly chosen to join the same number of zygotes from the other two dishes in the vessels that were set up to monitor offspring viability under each of the two environments. (c) In Experiment 2, the sperm of the three males were mixed before fertilizations.

In this paper, we test the three main hypotheses underlying bet-hedging theory using a novel experimental framework that overcomes all of the problems highlighted above. Specifically, we apply a maternal half-sibling design to an externally fertilizing marine invertebrate—the sea urchin *Heliocidaris erythrogramma armigera*—in order to assign individual females to a polyandrous and monandrous mating strategy simultaneously. Using this design, we then assess geometric mean fitness of both mating strategies across multiple (simulated) generations. Furthermore, we experimentally separate bet-hedging from sexually selected processes to explore their relative importance in generating fitness differences between treatments. Our ensuing results show that there is scope for a polyandrous strategy to prevail over a monogamous strategy due to genetic quality/compatibility benefits and genetic diversity benefits derived from a pure bet-hedging mechanism. The results also reveal the importance of direct and indirect benefits derived from sexually selected processes in explaining polyandry. Our study's ultimate goal is not so much to document the benefits of multiple mating in the subject species, but to provide a framework and a proof-of-principle approach that may prove useful in future examinations of bet-hedging theory in the context of sexual interactions.

2. Material and methods

(a) Model system

The purple sea urchin *H. erythrogramma armigera* is a common invertebrate that plays a key ecological role in intertidal

communities in southern Australia [37]. This highly fecund broadcast spawner is ideally suited to our purposes; individuals are easily induced to spawn thousands of gametes, thus facilitating controlled *in vitro* fertilizations with high levels of replication [38,39]. Critically, these attributes make it possible to estimate the fitness of individual females assigned to both a monandrous and a polyandrous strategy using a maternal full-sib-half-sib design (see below). High numbers of gametes also means that estimations of variance in fertilization success and post-zygotic viability can be based on large sample sizes, thus ensuring that sampling errors have minimal impact on our ensuing analyses of within- and between-generation fitness. Details about animal collection and maintenance can be found in the electronic supplementary material.

(b) Bet-hedging in the absence of sexually selected processes (Experiment 1)

We employed a design that enabled us to assess the fitness of individual females assigned to both a monandrous and a polyandrous treatment. The experiment was run in blocks ($n = 12$), with each block comprising one female and nine males whose identity differed across blocks. For each block, the gametes of the same female were partitioned into 18 equally sized batches (approx. 500 eggs each; see the electronic supplementary material), corresponding to two 'mating strategy' treatments (monandry and polyandry), three different 'mating events' per treatment, and three replicate Petri dishes per 'mating event' and treatment where mixing of gametes (i.e. fertilizations) took place (figure 1a). The three mating events were used to simulate three reproductive episodes, each of them taking place in a different 'simulated generation' (henceforth 'generation') (see below and figure 1a). This design allowed us to simulate across-generations fitness outcomes when a same clonal

lineage (an individual female genotype) follows both strategies. In other words, the within-block mating events in our design represent families of offspring that an individual genotype could have produced had it mated either singly or multiply across three independent generations. The ultimate aim was to compare the fitness (W) between monandrous and polyandrous strategies across successive generations (thus providing a key test of the evolutionary potential of these two mating strategies). Fitness between generations (henceforth W_{BG}) is calculated as a geometric mean using the scores of fitness within generations (henceforth W_{WG}), which are calculated as arithmetic means of the scores of individuals (females) following the same strategy in each generation.

In the monandrous treatment, the eggs in each of the three fertilization replicates per generation were fertilized by only one male who was randomly chosen among the nine males in the block. For each block and generation, the same male chosen for fertilizations in the monandrous treatment was used together with another two randomly chosen males in the polyandrous treatment. Thus, three males fertilized the eggs allocated to the polyandrous treatment in each generation, but critically the fertilizations were performed using non-competitive assays so that we avoid biases in paternity attributable to sexual selection (i.e. sperm competition or female sperm selection; see Experiment 2 where sexual selection is incorporated into the design). All crosses within each block took place at the same time but generations were numbered arbitrarily from 1 to 3 for labelling purposes and to account for the fact that one male was shared between the monandrous and polyandrous treatment per generation.

One advantage of the species used in our study is that fertilization can be determined soon after the fusion of gametes (2 h after conception). This limits the possibility that fertilization estimates are compromised by post-zygotic mortality, for example if incompatibilities between parental haplotypes are expressed after fertilization [40]. Fertilization was assessed 2 h after sperm and eggs were mixed by examining 100 eggs from each fertilization dish (n fertilization dishes = 216). Fertilized eggs were classed as such if cell division had occurred [38,39]. At this stage, from each fertilization dish 20 fertilized eggs (zygotes) were randomly selected and combined with 20 zygotes from each of the other two fertilization dishes of the same generation and treatment type (figure 1b). This was done twice (i.e. 40 embryos were collected from each fertilization dish), and each set of 60 eggs (20 egg per fertilization dish \times three dishes) was placed in a plastic container (10 cm diameter; hereafter 'offspring viability vessel') with 100 ml of filtered seawater. Immediately after transferring the embryos, each of the two vessels from each generation and treatment type was assigned haphazardly to one of two environmental conditions: Environment A (pH 8; normal pH conditions for seawater) and Environment B (pH 7; 'acidic' environment). The acidic pH of the Environment B was set before embryo transfer but not maintained afterwards to avoid complete mortality (i.e. ceiling effects) [41]. Offspring viability was measured by counting the number of offspring that were alive in the containers 8 days after fertilization. The viability score for each offspring vessel was calculated as the proportion of surviving individuals in the sample. We strove to attain large sample sizes for the number of embryos transferred to each vessel and low variation among the number of embryos across vessels to minimize the effect of sample size variation on offspring viability scores. On rare occasions low fertilization rates in some fertilization trials prevented us from reaching the target of 40 embryos collected from each fertilization dish, but we always kept the number of embryos collected from each of the three fertilization dishes per generation and treatment constant, and altogether we were able to set up between 40 and 60 embryos in over 85% (123 out of 144) of the embryo containers. Overall, we determined offspring survival for 7334 offspring in Experiment 1 (mean number of embryos per offspring survival

vessel \pm s.e.: 50.1 ± 1.2 , $n = 144$ containers distributed across all blocks, environments, treatments and generations). Further details about *in vitro* fertilizations, environmental conditions and scoring offspring viability can be found in the electronic supplementary material, and for clarity we also summarize the features of the experimental design in the electronic supplementary material.

(c) Predictions

(i) Genetic bet-hedging hypothesis

This hypothesis relates to stable environmental conditions and thus it can be tested using the data within environments (A or B). The hypothesis predicts that within each environment the fitness between generations (geometric mean fitness calculated with offspring survival data) for the polyandrous strategy (Poly W_{BG}) should be higher than for the monandrous strategy (Mon W_{BG}). The analysis therefore uses the offspring viability scores of each female (from 1 to 12) and given generation (x , y or z) to calculate first $W_{\text{WG},x}$, $W_{\text{WG},y}$ and $W_{\text{WG},z}$ (arithmetic mean fitness) for the two mating strategies, and then Poly W_{BG} and Mon W_{BG} (figure 1a). The advantage or disadvantage of polyandry arising from bet-hedging can be then measured as the difference between these two geometric mean fitness scores (Poly W_{BG} - Mon W_{BG}). An important aspect of the analysis is that it requires Monte Carlo simulations (see the electronic supplementary material).

(ii) Genetic diversity bet-hedging hypothesis

This hypothesis also predicts higher geometric mean fitness for polyandrous strategists between generations, but in this case under unstable environmental conditions. This hypothesis can be tested using the data from both Environment A and Environment B. The rationale here is that polyandrous females facing unpredictable or changing environments may obtain genetic benefits as a result of an increase in the genetic diversity of the offspring (i.e. by producing an array of offspring genotypes) which leads to an increase in the probability that at least some of these offspring will be adapted to the environment they encounter. We tested this hypothesis in two ways. First, we envisage a scenario in which offspring experience fluctuating environments across generations (i.e. A for the first generation, B for the second and A again for the third, henceforth *fluctuating environments ABA analysis* (or vice versa, *fluctuating environment BAB analysis*)). Second, we investigated a scenario in which offspring disperse to two different environments in each generation (henceforth, *averaging across environments A + B analysis*).

(iii) Bet-hedging for direct benefits

We investigated the scope for polyandry to generate direct benefits through bet-hedging by looking at differences in fertilization rates between mating strategies.

(d) Bet-hedging plus sexually selected processes

(Experiment 2)

Within each block, all fertilization trials (even those for the polyandrous treatment) in Experiment 1 were performed using a single female's eggs and a single male's sperm. Thus, the multiple males' ejaculates never competed over the same set of eggs, and there was no competition for fertilizations or sperm selection. This explicitly excluded biases in fertilization attributable to sexual selection. Adaptations that normally facilitate cryptic female choice such as male-by-female gametic interactions might still affect fertilization outcomes even if ejaculates do not compete. However, ensuring that the fertilizations are carried out on a one male-one female basis translates into a bet-hedging benefit; sperm selection is not possible because we simply picked up

Table 1. Fitness advantage/disadvantage of polyandry (Poly W_{BG} –Mon W_{BG}) attributable to bet-hedging alone (Experiment 1) or to the joint action of bet-hedging and sexually selected processes (Experiment 2). The mean and 95% CL of the distribution of values for the advantage/disadvantage of a polyandrous mating strategy (Poly W_{BG} –Mon W_{BG}) following Monte Carlo simulations described in the Methods is shown ($n = 10\,000$ iterations). The advantage/disadvantage of polyandry is expressed as a percentage. Bold font indicates that the confidence interval does not include zero.

trait	Poly W_{BG} –Mon W_{BG} (%)	lower 95% CL	upper 95% CL
Experiment 1			
offspring viability. Environment A (pH 8)	+ 5.4	4.97	6.01
offspring viability. Environment B (pH 7)	– 4.8	–5.19	–4.42
offspring viability. Across environments (A + B)	+ 0.3	0.07	0.65
offspring viability. Fluctuating environments (ABA)	+1.7	–2.20	5.67
offspring viability. Fluctuating environments (BAB)	–1.7	–5.54	2.25
fertilization rates	– 7.8	–8.65	–6.14
Experiment 2			
offspring viability. Environment A (pH 8)	–0.3	–0.54	0.02
offspring viability. Environment B (pH 7)	+ 2.2	1.77	2.74
offspring viability. Across environments (A + B)	+ 1.0	0.75	1.26
offspring viability. Fluctuating environments (ABA)	+0.41	–2.79	3.54
offspring viability. Fluctuating environments (BAB)	+1.25	–2.10	4.66
fertilization rates	+ 12.8	12.46	13.47

fertilized eggs by that male from the dish (even if the female did not prefer that male's sperm) and we forced females to put their eggs in different baskets (males) that lead to different fates of the eggs. In other words, gametic incompatibilities may contribute to fertilization rates but in our experiment the potential benefit would result from bet-hedging based on the fact that some males are unsuitable for fertilizations. This is at the core of the bet-hedging rationale applied to mating strategies; polyandrous females spread the risk of mating with unsuitable mates (and they may be unsuitable for a variety of reasons including infertility or genetic incompatibilities). Second, in the offspring survival assays embryos sired by some males are expected to enjoy higher survival than others, e.g. if embryo survival is exclusively due to paternal genes but also if embryo survival is determined by male-by-female genetic incompatibilities. This again enters the bet-hedging domain; females would spread the risk of mating with unsuitable mates, and since each male contributed equally to the offspring population (20 embryos each male) we eliminated the possibility that sperm competition or mate choice (including sperm selection) leads to fertilization biases that contribute towards offspring survival. In short, the fitness difference between the two treatments in Experiment 1 could be largely attributable to bet-hedging mechanisms.

In Experiment 2, however, we were interested in assessing how bet-hedging and sexually selected processes potentially interact to generate polyandry benefits. In short, Experiment 2 uses the same design described in figure 1a but the sperm from the three males in each mating event within the polyandrous treatment were mixed prior to fertilization (10 ml from each male; see figure 1c and compare to figure 1b), instead of being placed unmixed into separate Petri dishes. In this way, variation in fertilization success among males (i.e. due to differences in sperm competitiveness), or variation in fertilization success attributable to females (e.g. due to sperm choice) or male-by-female gametic interactions [42,43], may contribute towards fitness. Thus, unlike Experiment 1, where paternity rates were standardized for each of the three males within each block, the paternity of the 60 zygotes randomly selected to investigate offspring survival in each polyandrous mating event would be biased according to differences in fertilization success among competing ejaculates.

We were able to set up the target of 60 zygotes in over 95% (138 out of 144) of the embryo containers. As before, this ensured that offspring viability scores (proportions) were not influenced by variation in the sample sizes from which these scores were obtained. Overall, we determined offspring survival for 8485 offspring in Experiment 2 (mean number of embryos per offspring viability vessel \pm s.e.: 58.9 ± 0.4 , $n = 144$ containers distributed across all blocks, environments, treatments and mating events). Further methodological details about Experiment 2 can be found in the electronic supplementary material. Analyses were identical to those carried out for Experiment 1.

3. Results

In the analysis of bet-hedging in the absence of sexually selected processes (Experiment 1) fertilization rates across all samples were low (mean \pm s.e.: $38.6 \pm 2.3\%$; total number of replicates across all females, mating strategies, mating events and fertilization trials = 216), and the percentage of offspring surviving to 8 days was also low ($31.4 \pm 1.6\%$; total number of replicates across all females, mating strategies, mating events and environments = 144). The between-generation fitness (W_{BG}) of a polyandrous strategy significantly exceeded that of a monandrous strategy within Environment A (table 1). By contrast, in the acidic Environment (B), W_{BG} was significantly greater in the monandrous treatment (table 1). When W_{BG} was estimated across environments (A + B analysis), our analyses revealed that W_{BG} was significantly greater for polyandrous females (table 1).

Multiple mating via bet-hedging resulted in no gain or loss in fitness under fluctuating environments (ABA or BAB analyses) compared to monogamous matings. Fertilization rates were significantly lower in the polyandrous treatment ($34.5 \pm 3.1\%$) compared to the monandrous treatment ($42.7 \pm 3.4\%$) (table 1).

In Experiment 2 (examination of genetic benefits under bet-hedging and sexually selected processes), fertilization rates

across all samples were moderate ($62.5 \pm 2.0\%$; total number of replicates across all females, mating strategies, mating events and fertilization trials = 213) and the percentage of offspring surviving to 8 days was low ($24.5 \pm 1.4\%$; total number of replicates across all females, mating strategies, mating events and environments = 144). When sexually selected processes were allowed to operate in addition to bet-hedging, direct (fertilization) benefits were clearly boosted under polyandry ($68.4 \pm 2.4\%$ versus $56.8 \pm 3.1\%$ under monandry) (table 1). Polyandry returned higher between-generation fitness gains in Environment B, but no significant advantage or disadvantage in Environment A (table 1). As in Experiment 1, polyandry yielded genetic diversity benefits (as estimated from offspring viability) across environments (A + B analysis) but no sizeable genetic benefits under fluctuating environmental conditions (ABA or BAB analyses).

4. Discussion

Our study enabled us to overcome many of the logistical difficulties in testing whether females that mate indiscriminately with more than one male increase their fitness through genetic bet-hedging, genetic diversity bet-hedging or bet-hedging for direct benefits. Our ensuing results confirm that bet-hedging, in addition to sexual selection, can lead to significant increases in geometric mean fitness for multiply mated females.

(a) Genetic bet-hedging in the absence of sexually selected processes

Our data show that the between-generation (geometric mean) fitness, measured through offspring survival, of an exclusive bet-hedging polyandrous strategy (i.e. in the absence of sexual selection) was significantly higher than that of a monandrous strategy in Environment A. The difference in geometric mean fitness between the two strategies (5.4%) is large enough to be evolutionarily significant. Specifically, if a population begins with 99% monandrous genotypes and 1% polyandrous genotypes (e.g. as a result of a mutation increasing the propensity of monandrous females to mate with multiple males), and assuming that there is no mixing of genotypes and no costs associated with polyandry, a 5.4% fitness advantage suggests that the multiple-mating genotype will exceed 50% of the population after just 84 generations and will exceed 99% after just 167 generations (see [36] for similar calculations). Thus, our results indicate that polyandry via exclusive bet-hedging mechanisms can offset extremely high costs associated with polyandry of around 5%. However, we found that the genetic advantage of polyandry via bet-hedging was dependent on context because multiple mating was disadvantageous in the acidic environment (Environment B). Ultimately, the likelihood of polyandry spreading in a stable population through benefits associated with bet-hedging will depend on several factors, including (i) the frequency of suitable/unsuitable male genotypes: the higher the frequency of unsuitable males, the more likely that bet-hedging will bring benefits; (ii) population size: the smaller the population the more likely that there is selection against stochasticity; (iii) remating costs: high mating costs may offset the benefits derived from bet-hedging; and (iv) population subdivision: polyandry via bet-hedging can provide substantial benefits in

spatially structured populations (Yasui & Garcia-Gonzalez 2014, unpublished data, and see below and [32,35,44]).

(b) Genetic diversity bet-hedging in the absence of sexually selected processes

The genetic diversity bet-hedging hypothesis for the evolution of polyandry predicts that by mating multiply, females produce an array of offspring phenotypes that maximizes the chances that at least some offspring survive when environmental conditions are unstable. The issue, however, is not so much to know whether multiple mating increases genetic diversity within a clutch, but whether this diversity translates into an increase in the fitness of mothers [26]. Furthermore, the real challenge is to demonstrate that this within-clutch enhanced genetic diversity works as a bet-hedging strategy, reducing variance in fitness across generations and consequently increasing evolutionary fitness at the expense of sacrificing expected mean fitness.

A number of studies have shown that female multiple mating entails genetic diversity benefits (see Introduction), but to our knowledge none of them has tested whether these benefits are acquired and manifested through bet-hedging *per se*. McLeod & Marshall [33] compared the magnitude of benefits in terms of offspring performance (hatching success and larval survival) arising from paternity-biasing mechanisms at fertilization, with those brought about as the result of having equal proportions of offspring sired by different males, in a polychaete worm (and see also [34] for a test in an ascidian). Offspring performance benefits were entirely explained by effects arising from having mixed broods *per se*, i.e. in the absence of paternity biases leading to unequal representation of offspring within a clutch [33]. These results were interpreted as genetic diversity effects potentially mediated by reduced competition for resources in mixed embryos cultures. However, the influence of environmental variation was not inspected in that study (that is, fitness was assessed within a stable environment), and it is possible that the higher fitness of offspring in mixed offspring families resulted from higher fitness of offspring sired by certain male genotypes, even if there was no paternity bias at fertilization. Thus, although their results are consistent with the existence of genetic diversity effects, genetic bet-hedging (as defined in this study; see Introduction, and [26]), cannot be ruled out. Moreover, McLeod & Marshall's [33] study did not inspect fitness within an intergenerational framework and so it is unclear if the benefits they exposed could be interpreted as bet-hedging (see also [34]). It should also be emphasized that bet-hedging is defined as a reduction of variance (or a reduction in the among-individuals correlation in performance [5]) coming at a cost of reduced mean, and the studies above show an increase in the arithmetic mean that is in principle not consistent with bet-hedging. Nevertheless, these studies clearly demonstrate the existence of benefits of polyandry that are linked to increases in genetic diversity and that are independent of paternity-biasing mechanisms.

The first convincing evidence that bet-hedging is plausible in the context of multiple mating came from a study in which, paradoxically, multiple mating by males not females was investigated [36]. By comparing variances between full-sib families and paternal half-sib families, both within a given environment and across environments, Fox & Rauter [36] showed that the variance in mean fitness was always lower

for offspring in half-sib families and consequently geometric mean fitness was greater in these families. The logic underlying Fox & Rauter's [36] approach is that the advantages of multiple mating should apply equally to males and females. While this is essentially true, it is however possible that fitness differences between paternal half-sib and full-sib families are determined to some extent by maternal (non-genetic) effects, e.g. differential maternal allocation on the basis of male quality assessed before copulation. Such maternal effects can increase diversity in the response (offspring performance) of half-sib paternal families. By contrast, our study controls for differential allocation as well as any other forms of maternal effects because eggs from same females are assigned to both the monandrous and polyandrous treatments.

The genetic diversity hypothesis for the evolution of polyandry has been supported with results from studies on social insects showing an association between polyandry and productivity or disease resistance [45]. However, maternal effects can account for this association, for instance, if queens in better condition are those that tend to mate multiply. Other studies have removed the possibility of maternal effects by using artificial insemination [46,47], but in this case genetic diversity effects were not isolated from paternity-biasing effects. These latter effects could potentially explain to a large extent the benefits of polyandrous females.

We distinguished between genetic bet-hedging and genetic diversity bet-hedging effects and separate these from paternity-biasing effects, while controlling for maternal effects that can confound the assessment of the relative importance of all these kinds of benefit. We tested the genetic diversity bet-hedging hypothesis in two ways. First, we compared the geometric mean fitness of polyandrous and monandrous strategies when in each generation offspring from a given family were raised in contrasting environments. This can be considered analogous to a situation in which females distribute their offspring among environments, for instance as it occurs in some insects through oviposition on several hosts. Our results from Experiment 1, in which we test such a scenario, provide support for the genetic diversity bet-hedging hypothesis. Geometric mean fitness (W_{BG}) for polyandrous strategists was significantly higher than the fitness of monandrous strategists, although the difference was slight (0.3%).

Second, we tested the scope for bet-hedging to provide genetic diversity benefits in a situation where offspring experience fluctuating environments across generations, such that the environmental conditions in any given generation are different to the environmental conditions encountered in the preceding generation. Our results revealed no evidence for benefits under these conditions, most probably because the benefits of polyandry through bet-hedging mechanisms are context-dependent, and also because in our test we did not use worst-case environmental conditions. 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 [41] and in our study neither of the environments was found to seriously impede offspring survival during early (hours) development. Selection against stochastic demography can be determined by environmental fluctuation with drastic effects on fitness. If environments fluctuate but variation in the detrimental effects of the environmental conditions upon fitness is small, bet-hedging adaptations will not be favoured [48]. The corollary, from the perspective of empirical investigations of bet-

hedging, is that even if bet-hedging has evolved in a population, an empirical test may not detect it if the test does not include worst-case environmental conditions [10]. Our results therefore need to be interpreted accordingly.

(c) Bet-hedging and sexually selected processes

In our second experiment, sexually selected processes were allowed to operate in addition to bet-hedging. Under this scenario, polyandry returned higher geometric mean fitness than monandry through increases in offspring survival in Environment B (2.2% advantage of polyandry). Given the lack of bet-hedging effects in Environment B in our first experiment, the advantage of polyandry through genetic benefits in Experiment 2 is probably attributable to sexual selection [38,39,42]. Polyandry resulted in genetic diversity benefits when averaging across environments, as in Experiment 1, but in this case the advantage of polyandry (1.0%) was higher than when sexually selected processes were prevented from operating. Thus, overall our results suggest that sexual selection augments the fitness payoffs of multiple mating as far as genetic and genetic diversity benefits (and direct benefits too; see below) are concerned.

We investigated whether bet-hedging and sexually selected processes enable females to obtain direct benefits in the form of insurance against infertility problems [49]. Consistent with previous results on this species [39,43], we found that males vary in their ability to fertilize ova as evidenced by strong male effects at fertilization (see electronic supplementary material: supplementary results and table S1). Our results indicate that paternity-biasing mechanisms play a more prominent role than bet-hedging in the accrual of these direct benefits by multiply mated females. When both bet-hedging and sexually selected processes were allowed to operate the polyandrous strategy returned a 12.8% fitness advantage measured at fertilization. Such advantage is clearly substantial; a multiple-mating mutant starting with a 1% frequency in a population would reach a frequency more than 50% in just 35 generations. Our results thus indicate that the deterministic effects of sexual selection delivers direct fertilization benefits, which can further bolster the genetic benefits of polyandry. They additionally suggest that both direct and indirect benefits arising from these deterministic effects can work along selection against stochasticity (i.e. bet-hedging), or compensate for the cost of such selection.

(d) Conditions for the evolution of polyandry via bet-hedging

Bet-hedging in the context of multiple mating can be considered as within-generation bet-hedging (after [50,51]). In within-generation bet-hedging environmental heterogeneity is fine-grained (different environmental conditions affect individuals of the same generation) in contrast to between-generation bet-hedging, where environment is coarse-grained (all individuals within each generation experience the same environmental conditions). In the case of multiple mating, all females in the population in any given generation can mate either monandrously or polyandrously. Coarse-grain environments creates more scope for selection against variance than fine-grain environments because in the former all individuals experience the same conditions and thus if the conditions in a given generation are bad for a certain genotype, lineage or strategy (e.g. a dry year affecting a wet year

specialist), that lineage would see its fitness greatly reduced or would even become extinct [5]. Coarse-grain environmental heterogeneity thus creates the condition for the evolution of bet-hedging more easily than fine-grain environments, where not all bet-hedgers with the same phenotype experience the same fate [51]. Fine-grain environmental variation is unlikely to favour the evolution of bet-hedging in large populations, because in within-generation bet-hedging the effects of variance reduction are largely diminished by population size [5,6,26,32,50,51].

So, what are the chances that bet-hedging plays a role in the evolution of polyandry? The answer is not simple. In large populations, the 'bad-luck' of monandrous females paired with an unsuitable male would be compensated with the 'good-luck' of monandrous females paired with suitable males and the fitness advantage of bet-hedgers may be so small that even small costs associated with multiple mating will make bet-hedging mechanisms alone unlikely to explain the evolution of multiple mating [5,26,32,51]. However, populations in nature are frequently spatially structured and individuals occur in subpopulations more or less interconnected by migration. Under these conditions, bet-hedging can work to promote the evolution of multiple mating (Yasui & Garcia-Gonzalez, unpublished data, and see also [35]). In general, the evolution of bet-hedging strategies is favoured when populations are subdivided in local demes [11,44,52]. Even in large populations, mating may take place locally before or after individuals migrate among patches. Also, it is now becoming evident that populations can be structured as a result of non-random behavioural interactions (behavioural subdivision). Furthermore, it may be that at least in some animals the costs of multiple mating are small. We also know little about how benefits attributable to bet-hedging and sexual selection combine or act synergistically (but see Results). If the two mechanisms act synergistically, it is possible that the combined benefits can compensate for very large costs of mating. Nevertheless, the relative importance of bet-hedging and paternity-biasing mechanisms in the evolution of polyandry would have to be weighed against other mechanisms, such as the existence of shared genetic basis for mating behaviour across sexes, or convenience polyandry in the face of sexual conflict (see Introduction). Future studies in a range of taxa teasing apart these different, but presumably non-mutually exclusive,

explanations for the evolutionary maintenance of polyandry are warranted.

(e) Concluding remarks

Our analyses enable us to conclude that bet-hedging may be an important factor underlying the evolution of female multiple mating because of benefits associated with risk spreading that do not require reliable or even existent mate quality assessment. Our paper also illustrates difficulties in testing bet-hedging theory, and thus highlights the scarcity of empirical tests in the context of multiple mating. Nevertheless, we anticipate that our suggested empirical approaches will provide a useful point of departure for work that will enable researchers to overcome many of the difficulties in testing bet-hedging theory. Our empirical results suggest that there is scope for females to increase fitness through genetic benefits when they follow a multiple mating bet-hedging strategy, and they further reveal that sexual selection can augment these benefits. These results call for an increased effort in understanding the mechanisms leading to stable polyandrous behaviour. We hope that this work spurs future investigations integrating the mechanisms and ecological factors (including the role of environmental fluctuation) that underlie the evolution of polyandry.

Data accessibility. Data are available from the Dryad Digital Repository: <http://doi.org/10.5061/dryad.v6n86> [53].

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