

MULTIPLE MATING IN THE GLANVILLE FRITILLARY BUTTERFLY: A CASE OF WITHIN-GENERATION BET HEDGING?

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Many hypotheses have been proposed to explain multiple mating in females. One of them is bet hedging, that is avoiding having no or very few offspring in any given generation, rather than maximizing the expected number of offspring. However, within-generation bet hedging is generally believed to be an unimportant evolutionary force, except in very small populations. In this study, we derive predictions of the bet-hedging hypothesis for a case in which local insect populations are often small, offspring performance varies, for example, due to inbreeding depression, and the groups of gregarious larvae have to exceed a threshold size before they are likely to survive throughout the larval stage. These conditions exist for populations of the Glanville fritillary butterfly (*Melitaea cinxia*), potentially making bet-hedging benefits larger than usual. We observed matings in a field cage, which allowed detailed observations under practically natural conditions, and analyzed genetic paternity of egg clutches laid by females under direct observation. The egg-laying and survival patterns are in line with the predictions, supporting the hypothesis that multiple mating in *M. cinxia* presents a rare case of within-generation bet hedging.

KEY WORDS: Compatibility, genetic bet hedging, inbreeding, indirect genetic benefits, last-male precedence pattern, *Melitaea cinxia*, metapopulation, microsatellite, multiple mating.

Females mate multiply in a wide range of taxa (Birkhead and Møller 1998). Whereas for males reproductive success is expected to increase linearly with the number of mates, the advantages of multiple mating for females are less clear (Yasui 1997; Jennions and Petrie 2000). Mating can be costly to females in terms of time and energy, or because of increased risk of predation, injury or infection (Chapman et al. 1995; Blanckenhorn et al. 2002). Multiple mating by females has been explained in terms of direct benefits, particularly in species in which males provide females with a nutrient-rich ejaculate (Arnqvist and Nilsson 2000) or in which resisting mating attempts would be too costly (costs represent the flipside of direct benefits (e.g., see Lee and Hays 2004), and in terms of indirect genetic benefits (e.g., see Newcomer et al. 1999; Jennions and Petrie 2000; Fedorka and Mousseau 2002; Kozielska et al. 2004).

Finally, genetic bet hedging (Gillespie 1973, 1974, 1975, 1977; Seger and Brockman 1987; Hopper 1999) could explain polyandry, especially when females mate indiscriminately (Yasui 1998, 2001; Fox and Rauter 2003). Multiple mating is a form of bet hedging because it creates clutches (or groups of clutches) with mixed parentage, as opposed to the monandrous case where a female picks one male to sire all the offspring in all of her clutches. Bet hedging is often used to describe “adaptive coin-flipping” (Kaplan and Cooper 1984), but in general it is a strategy that reduces the variance in fitness among offspring. It is usually defined in such a way that the mean fitness decreases, and thus variance reduction is the only benefit (Yasui 1998; Jennions and Petrie 2000). In the context of polyandry, the intuitive appeal of bet hedging is that even if polyandrous females cannot bias paternity patterns, they can hope to avoid

the worst-case scenario where all their offspring are fathered by a low-quality or an incompatible (e.g., closely related) male (Jennions and Petrie 2000; Fox and Rauter 2003). This type of bet hedging serves to avoid the detrimental effects of demographic stochasticity.

However, avoiding worst-case scenarios is not a guarantee of evolutionary success. Current theory predicts that bet hedging is far more likely to be a successful evolutionary strategy when the bets are hedged over several generations, than in a within-generation scenario, to which polyandry belongs (Yasui 1998; Hopper and Rosenheim 2003). In the latter case, the benefits of bet-hedging are predicted to vanish in all but the smallest populations (Yasui 1998; Hopper and Rosenheim 2003).

The Glanville fritillary butterfly (*Melitaea cinxia*) exists in the Åland Islands in southwestern Finland as a large metapopulation (Hanski 1999; Nieminen et al. 2004). Females mate usually only once, or occasionally twice (Boggs and Nieminen 2004). In 167 and 131 wild-caught mated females, only 8% and 6.5% had mated twice based on the count of spermatophores (Kuussaari 1998). In this species, direct benefits of multiple mating are unlikely (see Discussion), but there is a high level of inbreeding and substantial inbreeding depression in small local populations (Haikola et al. 2001; Nieminen et al. 2001). Nevertheless, females do not discriminate against close kin as mates (Haikola et al. 2004), possibly because the cost of such discrimination would be too high when there are often very few males locally available (Kokko and Mappes 2005).

To test whether the bet-hedging hypothesis is likely to explain the occurrence of polyandry in the Glanville fritillary, we first created the theoretical set of conditions and predictions that the system has to show for bet hedging to apply, and then tested these by determining the reproductive success of singly or multiply mated *M. cinxia*, using material that was collected by intensively observing an experimental population of ca. 200 butterflies of equal sex ratio in a large cage in the field (Hanski et al. 2006). This allowed us to quantify multiple mating patterns more precisely than in the field, to detect any postcopulatory paternity-biasing mechanisms, and to tract the number of larvae that survive until diapause.

Deriving the Predictions and Conditions of the Bet-Hedging Hypothesis

The precise list of predictions made by the bet-hedging hypothesis through polyandry is not readily available from the literature. We shall therefore derive the relevant conditions and predictions here, taking into account the relevant biological features of *M. cinxia*. There are two a priori reasons why bet-hedging could be important in this butterfly. Firstly, it persists in the Åland Islands as a metapopulation, in which no single popula-

tion is safe from extinction within a short period of time (Hanski 1999; Nieminen et al. 2004). Single populations are ephemeral, and they are mostly very small, up to the point where a single mated female has established an entire local population if she has mated and lands in an empty patch. This means that evolution favors a reduction in variance in individual reproductive output.

The second reason is related to the overwintering habits of *M. cinxia*. Females lay their eggs in clusters, and the larvae spin a web on the host plants on which they live. The larvae diapause as a group, and they tend to remain gregarious until the last molt before pupating (Kuussaari 1998). An important cause of mortality in natural populations is overwinter mortality; 20% of the larval groups die during the winter. Overwinter mortality is known to be dependent on larval group size, and small groups of less than 25 larvae have a very low chance to survive (Kuussaari 1998; Nieminen et al. 2001), probably because small groups are unable to build a high-quality winter nest, which is necessary for successful overwintering (Nieminen et al. 2001). Based on the data in Kuussaari (1998), we constructed a logistic regression of the probability of overwintering survival as a function of group size; the survival probability increases sharply with the number of prediapause larvae (Fig. 1). This result implies that prediapause groups of less than 25 larvae have low overwintering chances; we call this the Allee threshold size for larval groups.

These two observations both improve the prospects of a bet-hedging strategy. Before we derive the predictions and conditions, let us consider a simple illustrative example where a hypothetical female butterfly can lay 100 eggs. To do this she may mate monandrously, polyandrously with two males such that there is no mixed paternity within one clutch, or polyandrously with two males such that half of offspring in each clutch are fathered by each male. We consider an illustrative case with two very different male types. One is largely unsuitable as a mate, for example, due to extreme inbreeding depression: he gives offspring who survive from summer till diapause with a very low probability $s_1 = 0.05$, the other one yields survival $s_2 = 0.5$. All strategies give the same mean of offspring production (27.5 in this example), but the variances differ strongly.

Under monandry, the expected number of surviving offspring is strongly bimodal, with variance 521.13. Mixed-paternity polyandry yields a drastic reduction in variance (270.53): in half of the cases she mates with two different males, yielding a peak of intermediate survival (Fig. 2). The distribution produced by the no-mixed paternity case depends on the number of clutches. If all eggs are laid in a single clutch, one of the males yields no paternity at all, and the situation is identical to monandry. However, if there are two clutches, and the latter may (with 50% probability) be fathered by a different male, and the outcome is very similar to

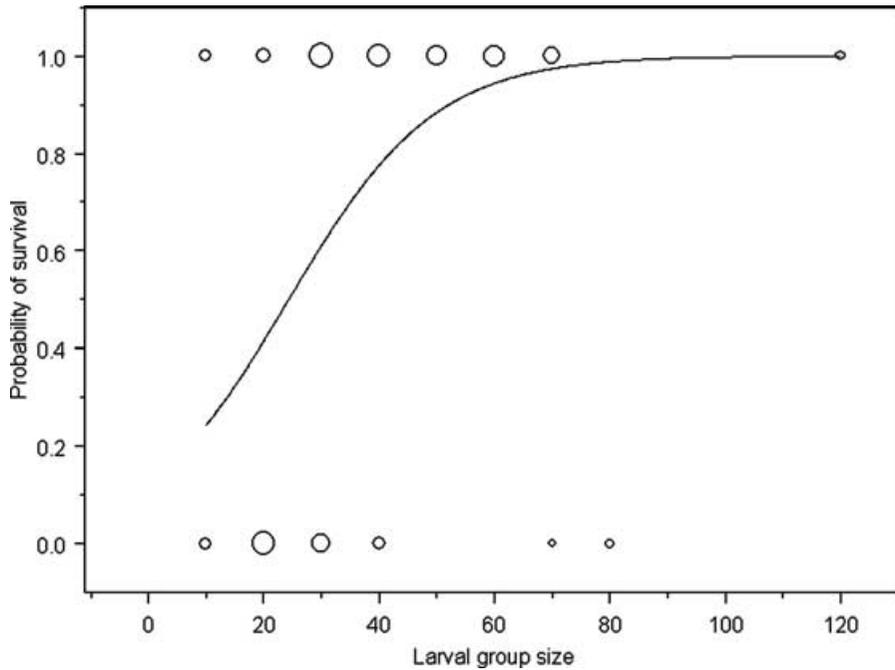


Figure 1. Logistic regression of the probability that a clutch survives the winter, from data presented in (Kuussaari 1998). The regression is given by $(\text{probability of survival}) = [1 + \exp(1.9219 - 0.079n)]^{-1}$, when the prediapause nest contains n larvae ($\beta = 0.079$, $\chi^2 = 19.83$, $df = 1$, $P < 0.001$). Below the Allee threshold of 25 larvae, successful overwintering is highly unlikely.

the case of mixed paternity (Fig. 2). The variance in this case is the lowest of all three strategies (268.00 in the example of Fig. 2), and the probability of complete failure (no offspring produced at all) is halved relative to monandry.

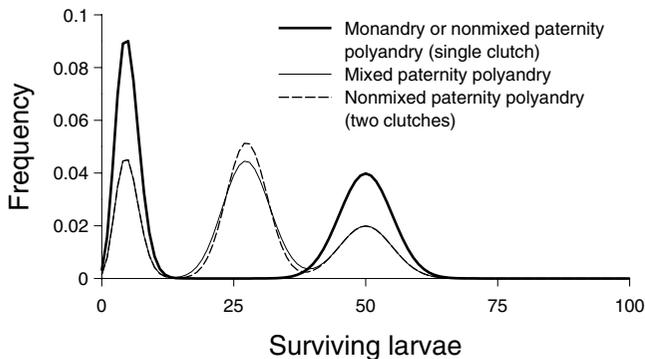


Figure 2. A hypothetical example where a female has 100 eggs, and there are two equally common male types, giving offspring with survival $s_1 = 0.05$ or $s_2 = 0.5$. The survival of each offspring until diapause is assumed to be independent of the survival of others, and neither monandrous nor polyandrous females can detect male type. Thus polyandrous females are assumed to mate twice but in 50% of cases this means remating with the same male type. Polyandrous females can opt to lay two clutches 50 eggs each (one for each mate) instead of one clutch, though the result only differs in the case of no-mixed paternity polyandry. See text for details on the variances in the number of surviving offspring.

All these effects arise without incorporating the Allee threshold. If overwintering survival chances of offspring increase nonlinearly with the number of surviving larvae, the outcomes will depend strongly on the division of larvae into larval groups: it is important to have the bulk of the probability distribution of each clutch lie above the threshold, but if overwintering mortality hits most of larvae in one clutch, it may also be important to lay several clutches to reduce the variance caused by destruction of whole winter nests, which leads to the demise of all larvae inside (Fig. 1). Because this trade-off between individual clutch sizes and the total number of clutches is difficult to visualize in Figure 2, we will now leave this simplified example and turn our attention to a more systematic investigation of possible fitness consequences with different mating and clutch size strategies.

Figure 3 examines predictions for five different fitness components for females, who mate singly, multiply but with only one sire per clutch, and multiply with mixed paternity (two males with 50% each) in each clutch. Fitness components are derived assuming that there are two types of males, and the prediapause survival of each egg (i.e., from egg-laying until the overwintering nest is built) is s_1 if the sire is of type 1 and s_2 if the sire is of type 2. As before, these could reflect, for example, related and unrelated males, the former yielding low survival of offspring due to inbreeding depression (Keller and Waller 2002; Haikola et al. 2004). The prediapause survival of each egg is assumed to be independent of the survival of other eggs, thus the number of

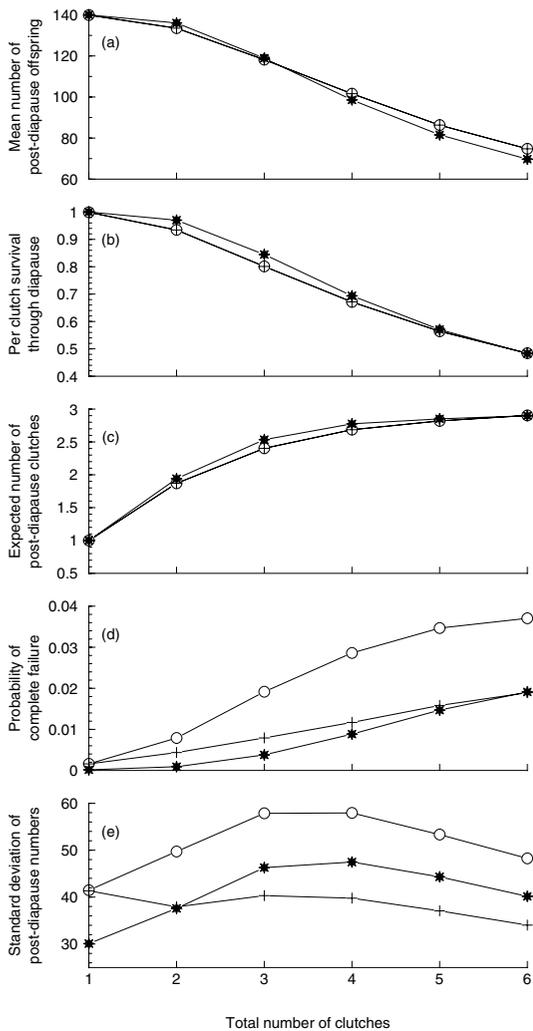


Figure 3. Predicted values of various fitness components for different mating strategies. We assume two potential sires, one giving survival from egg to diapause s_1 for each egg, the other s_2 . Fitness components are then calculated assuming monandry (open circles), polyandry with no mixed paternity (crosses), and polyandry with each male siring 50% of offspring (stars), and the total egg budget is 400 eggs for each female, divided into one to six clutches. The mean number of postdiapause offspring (a) is obtained by first computing the weighted sum of binomial distributions of the number of prediapause offspring (weights indicate the probability of mating with either type of male, e.g., a monandrous butterfly has 50% of chance of survival s_1 for each egg and 50% of s_2), and then assuming that each clutch survives the winter independently from other clutches with a probability that depends on clutch size (b), as indicated in the logistic regression of Fig. 1. Both the expected number of surviving postdiapause clutches (c) and the probability of complete failure (d) (the probability that there are no clutches that survive the winter) increase with the number of clutches. Variance (e) (indicated as standard deviation of the number of postdiapause larvae) is reduced by bet hedging, but in the case of no mixing of paternity, this requires increasing the clutch size. The examples are derived using $s_1 = 0.25$, $s_2 = 0.45$.

prediapause larvae is binomially distributed. This assumption is not entirely true, as there is in *M. cinxia* a consistently positive effect of group size on survival throughout development from egg stage to the last caterpillar instar (Kuussaari et al. 2004). However, due to scarcity of data we have not quantified this relationship, and assuming density independence will give us conservative estimates of the prospects of bet hedging (as including it would add another Allee effect). Survival through diapause is then assumed to depend on the presence of other larvae as in Figure 1. In the examples of Figure 3, a female is assumed to have a total budget of 400 eggs, and she can lay them in one to six equal clutches (i.e., in batches of 400, 200, 133, 100, 80, or 67 eggs, respectively).

Regardless of the mating strategy, the simple arithmetic mean of postdiapause offspring number decreases with an increasing number of clutches (Fig. 3a), together with the decline in the survival of individual clutches (Fig. 3b) due to the smaller size of each individual clutch. However, the expected number of surviving clutches increases when there are many clutches (Fig. 3c). Such “safety in numbers” in terms of numbers of clutches shows a trade-off with “safety in numbers” operating within a clutch. The probability of complete failure, that is, no surviving postdiapause offspring, increases with the number of clutches which the eggs are divided into (Fig. 3d), reflecting the intense within-group Allee effect of overwintering survival in small groups. Finally, multiple mating reduces the variance in postdiapause offspring numbers significantly (Fig. 3e), but to achieve this effect when paternity is not mixed, the female should also decrease her clutch size.

In a species with population dynamics as complicated as in *M. cinxia* (Hanski et al. 1995) it is not easy to determine which fitness component best reflects success in nature: maximizing those in Figure 3a–c, minimizing complete failure, Figure 3d, or minimizing variance, Figure 3e. In reality, the best strategy is likely to balance some of the benefits of each measure. Keeping this in mind, we can obtain several qualitatively robust results from Figure 3. In the following we list these as two “predictions” and three “conditions” that must be met for bet hedging to apply.

If bet hedging is unimportant and individuals simply maximize the expected number of postdiapause offspring, selection always favors laying eggs in as few and large clutches as possible (Fig. 3a), where “as possible” should be considered taking into account physiological limits and local availability of larval food sources. However, if clutches also experience random mortality (irrespective of their size) that kills all offspring in one clutch, it should become beneficial to bet hedge by enlarging the expected number of clutches that survive until the next generation. Bet-hedging thus predicts that clutch sizes will reflect a trade-off between the decrease in Figure 3a and the increase in Figure 3c, that is, some intermediate choice. However, this type of bet hedging relies on random (independent) mortality across clutches, and does not predict that the clutch sizes of monandrous

and polyandrous females would differ: the fitness components do not differ much between the mating strategies. We thus arrive at our first prediction:

Prediction 1. If females do not bet hedge at all, they should lay as large clutches as possible given the constraints that operate on, for example, female physiology and larval ecology. But if they bet hedge because of random mortality hitting individual clutches, they should lay more and smaller clutches. This prediction applies across all mating strategies.

Prediction 1 may be hard to test because it is difficult to know about all potential constraints, and because it does not predict strong differences between female mating strategies. In contrast, the mating strategy has a large effect on the probability of total failure (Fig. 3d) and on the variance in the number of postdiapause offspring (Fig. 3e). Monandrous females experience a much greater probability of failure, in particular if they lay many small clutches. All their clutches are necessarily genetically similar, and if they have mated with a male who yields few surviving offspring, then all clutches are likely to fail due to the within-group Allee effect. They can thus only avoid a significant risk of complete failure (Fig. 3d) if they lay many eggs in a single clutch (in the hope that enough survive above the Allee threshold). For multiply mating females that mix paternity within broods, the risk of total failure is smaller (Fig. 3d) and they can achieve low variance in the number of postdiapause offspring even if they do not lay many clutches (Fig. 3e). If mixing paternity is not an option, however, females need to gain the bet-hedging benefit by laying many clutches: some will then be sired by very suitable males, others by very unsuitable ones, and the total risk of going below the Allee threshold in every clutch remains small.

It follows that if the fitness components of minimizing total failure or the variance are important, we predict them to have a much stronger selection for monandrous females to increase their clutch sizes than for polyandrous ones, and polyandrous females are instead selected to increase the number of clutches particularly if they cannot mix sperm within clutches. The risk reduction through multiple mating frees these females to reap the advantages of other fitness components, such as that outlined in prediction 1. We arrive at our second prediction:

Prediction 2. If bet hedging to avoid the within-group Allee effect is an important component of fitness, we predict differences between mating strategies in the size and number of clutches. Monandrous females should lay few large clutches, whereas polyandrous females gain the bet-hedging benefit by laying several small clutches. The difference between polyandrous females that mix paternity within clutches and those that do not is predicted to be small, but in the case of a difference, females that produce clutches of nonmixed paternity should have the smallest clutches.

Figure 3 illustrates these predictions using single numerical examples only, but other numerical choices lead to qualitatively identical conclusions: in particular, we created 100 random values for s_1 and s_2 between 0.05 and 0.95, and checked the number of clutches that minimizes the variance for each mating strategy. Monandrous females were always favored to lay a smaller (64% of cases) or identical (36%) number of clutches than females with nonmixed paternity polyandry, never a larger one. Mixed-paternity polyandry had more variable optima, which were below the optimum of monandrous females in 18% of cases, identical to them in 74% of cases, and larger in 8%. Thus the mixed-paternity strategy in general yields a clearer prediction regarding variance reduction than a directional selection on clutch size (Fig. 3e), whereas our prediction that differences in clutch size strategy reflect differences in Figure 3d–e remains very robust if females usually cannot mix paternity within broods.

All fitness components become identical across all mating strategies if $s_1 = s_2$. This result relates to the first of three additional conditions that the mating system has to fulfill for the bet-hedging hypothesis to apply:

Condition 1. Explaining multiple mating through bet-hedging requires showing that males vary in their ability to produce viable offspring with a given female.

Note that this does not require that males can be ranked in an order of genetic quality; compatibility issues such as inbreeding, which vary from female to female, are sufficient.

There are two additional, general conditions of the bet-hedging hypothesis:

Condition 2. Polyandrous females should have a lower variance in the number of surviving offspring.

This is a direct reflection of the nature of the bet-hedging hypothesis in general (Yasui 1998).

Condition 3. All other factors being equal, polyandrous females should run a lower risk of all their clutches falling below the Allee threshold.

This last condition may be difficult to test, because all other factors are not expected to be equal. If monandrous females optimize their reproduction and lay fewer clutches, the net risk for both types of females can become rather similar (e.g., compare the three clutches for monandrous females with six for polyandrous, Fig. 3d). However, if multiple mating evolves for the reason that it reduces the risk of total failure, it should logically retain a net benefit.

Material and Methods

FIELD CAGE EXPERIMENT

Postdiapause larvae were collected in the spring 2003 from 40 local populations in the Åland Islands in southwestern Finland and reared under common garden conditions in the laboratory.

Pupal weight was used as a measure of body size. Altogether 194 newly enclosed butterflies (81 females and 113 males) were sexed, marked, and released into a $32 \times 26 \times 3 \text{ m}^3$ field cage covered with mesh. The cage had been constructed on a natural dry meadow. The cage and the experiment conducted in it are described in (Hanski et al. 2006). Natural flowers existing at the site provided nectar to butterflies, whereas 250 larval host plants (*Plantago lanceolata*) in flower pots were placed in the cage for ovipositing females. Prior to their release, a small piece was removed from the hind wings of each butterfly for DNA analysis. Matings and ovipositions were carefully recorded, but a small fraction was nonetheless missed, which became evident as the host plants were checked every evening for egg clutches. The egg clutches were removed immediately after oviposition, and they were reared in the laboratory until diapause. Larvae were counted soon after hatching to determine egg hatching rate, and they were counted again just before diapause to determine prediapause larval survival. The fact that the eggs were brought into the laboratory for hatching and rearing removes all environmental effects except for maternal–paternal pairings, a necessary condition for evaluating whether males vary in their ability to sire young.

Out of the 67 females that were recorded to lay fertile eggs, 18 had not been observed to mate (Hanski et al. 2006). Most of the ovipositions were detected, as only 32 additional egg clutches were discovered by inspecting the host plants in the evening, whereas 225 ovipositions were recorded directly. Multiple mating was observed relatively frequently: 14 females were recorded mating twice, and four females mated three times. Thirty-one females were observed to mate once.

PARENTAGE ANALYSIS

All the 194 butterflies that were released into the cage were genotyped. Of the 18 females that had been recorded to mate multiply, 11 females produced enough offspring after the second mating to be used for parentage analysis. Altogether 354 larvae from 32 clutches laid by these 11 females were analyzed. For each clutch, five to 12 larvae were genotyped. Genomic DNA was extracted and amplified with polymerase chain reaction (PCR) using the primers described by (Sarhan 2006). Details of the PCR protocols and the cloning and characterization of the microsatellite loci are given in Sarhan (2006). The PCR products were run on an ABI Prism 377 automated sequencer (Perkin-Elmer) and the alleles were scored using the Genotyper software (Applied Biosystems). Amplification products from five primers allowed unambiguous paternity assignments.

DATA ANALYSIS

The data were analyzed using generalized linear models using the R Software (RDevelopmentCoreTeam 2005). The significance of a variable was tested based on the difference in deviance between

a model with and without that variable, using *F*-tests. All females that had not been observed mating but produced eggs that hatched successfully were considered monandrous (unmated females can produce only sterile eggs). They were obviously not included while evaluating the significance of male identity. For some of the egg clutches, the number of hatched larvae had not been recorded, and these clutches were removed from the analysis of late-larval survival. Out of the 18 polyandrous females, two females had to be removed from all analyses because of missing records of the number of diapause larvae.

When analyzing the influence of female mating status (monandrous vs. polyandrous) on clutch size, first clutch size (when laid before remating), and best clutch size, we assumed a quasi-Poisson distribution. The influence of female mating type on the size of the largest clutch produced was evaluated by comparing a model with that variable to a model where the life-time egg production was the only explanatory variable. In the analyses of clutch number, life-time egg production, number of diapause larvae per clutch, and total number of diapause larvae, all egg clutches produced by the same female were pooled, and we assumed a quasi-Poisson distribution. For the analysis of egg hatching rate, late-larval survival (survival from hatched larvae until diapause), total larval survival (from egg to diapause larvae), female remating propensity, and likelihood for a male to be chosen by a mated female, we assumed a quasi-binomial distribution. Because females produce several egg clutches during their life time that tend to become smaller with time, female identity was used as a random factor and clutch rank was used as an independent variable in all analyses where egg clutches were not pooled, and the significance of female mating type was evaluated based on the difference between a model with and without that variable. Similarly, the influence of male identity on the same dependent variables was evaluated by comparing a model including this variable to a model with only female identity and clutch rank as independent variables.

Finally, the total number of diapause larvae was regressed against life-time egg production separately for monandrous and polyandrous females, and an *F*-test to compare two variances was used to test for a difference in variance in the residuals between monandrous and polyandrous females.

To test whether males are sperm limited, we analyzed whether the number of times a male had mated previously and the time since its last mating had an influence on the total number of eggs the female will produce, the egg hatching rate and larval survival, and whether the female will remate. To assess the trading up hypothesis, we analyzed whether males that mated females chose to remate with were different from other males in the population (age, weight, total number of matings achieved, total number of offspring produced). We also tested, using logistic regression, whether the probability that the clutch exceeds the Allee

threshold at the prediapause stage depends on the female's mating status.

Results

PARENTAGE ANALYSIS

Out of the 32 polyandrous clutches analyzed, four were produced before the second mating, and hence the first male was the only possible father. This was confirmed by the genetic analysis. In 18 clutches out of the 28 remaining, the last male to mate with the female fathered all of the offspring. This includes all three clutches that were produced by females after third matings. For one egg clutch, the genetic analysis revealed that it had mistakenly been attributed to a particular female. This egg clutch was removed from all further analyses. There were nine egg clutches produced by five females that were not entirely sired by the last male they mated with. These exceptions to the last-male sperm precedence could not be explained by the age of the males, number and timing of previous matings, or time span between matings.

MULTIPLE MATINGS: TESTING THE PREDICTIONS

The total number of eggs produced did not differ significantly between monandrous and polyandrous females (404.16 ± 237.74 and 401 ± 215.66 , respectively, $F_{1,63} = 0.002$, $P = 0.963$). Egg-hatching rate and offspring survival were not affected by the female's number of matings: mean offspring survival from egg to diapause was similar for both groups ($F_{1,66} = 1.53$, $P = 0.22$). There was no difference between monandrous and polyandrous females in the total number of offspring surviving until diapause (131.35 ± 101.90 and 147.5 ± 91.72 , respectively, $F_{1,63} = 0.309$, $P = 0.580$).

Polyandrous females produced smaller clutches than monandrous females (90.14 ± 62.92 and 135.64 ± 63.15 , respectively, $F_{1,66} = 6.34$, $P = 0.014$) and they divided their fecundity into significantly more clutches than monandrous females (4.38 ± 2.99 and 2.98 ± 1.57 , respectively, $F_{1,63} = 5.94$, $P = 0.018$). Females that produced clutches with mixed paternity were not significantly different in this respect from polyandrous females that were not recorded to have mixed clutches, although the sample size is small with only three females with mixed paternity (altogether six clutches). In 12 out of 16 cases the female who eventually became polyandrous had not yet mated more than once when producing the first clutch, and they laid smaller first clutches than monandrous females (122.9 ± 67.59 and 169.4 ± 63.19 , $F_{1,59} = 5.007$, $P = 0.029$).

Clutches of females whose matings were observed could be classified in five categories: (1) laid by a singly mated female ($n = 87$); (2) laid by a multiply mated female, 100% of eggs fertilized by the first male ($n = 20$); (3) laid by a multiply mated female, 100% of eggs fertilized by a later male than the first ($n =$

15); (4) clutch laid by a multiply mated female, containing mixed paternity ($n = 6$); and (5) laid by a multiply mated female, and paternity distribution unknown ($n = 33$). Pooling all clutches within each category, the probability of exceeding the Allee threshold (at least 25 offsprings surviving until prediapause stage) increased significantly with clutch size in each category (Fig. 4). Including female identity as a random factor (generalized linear mixed model with binomial error), we could not detect significant differences between the logistic regressions, apart from a nonsignificant tendency ($P = 0.06$) that clutches with 100% paternity by a later male exceed the Allee threshold more easily than those sired by the first male of a polyandrous female (statistics given in Fig. 4). Applying a Bonferroni correction would move this value of P further away from significance.

To test the prediction that the variance in the number of surviving offspring is smaller for polyandrous females, we needed to correct for the fact that variance increases with the number of eggs laid. The variance of the residuals in number of offspring surviving until diapause (regressed against eggs laid) was higher for females that had mated only once relative to females that had mated several times (Fig. 5a, F -test to compare two variances: $F_{15,48} = 0.37$, $P = 0.04$). The number of offspring surviving in the female's best clutch was higher in females that had mated several times than in females that had mated only once, for any given clutch size (Fig. 5b, $F_{1,63} = 6.806$, $P = 0.011$). Out of 16 multiply mated females, none failed to produce at least one prediapause clutch that exceeded the Allee threshold. Of the monandrous females, this number was six out of 66 (9.1%). This is a nonsignificant trend in the predicted direction (Fisher's exact test, one-tailed $P = 0.26$).

Male identity had an influence on the number of hatched larvae ($F_{12,97} = 3.63$, $P = 0.0002$), and the number of diapause larvae ($F_{12,107} = 2.91$, $P = 0.002$), and it also influenced the egg hatching rate ($F_{12,97} = 3.56$, $P = 0.0003$). Although male identity had no influence on late larval survival (i.e., survival from hatched larvae to diapause larvae) $F_{10,82} = 0.51$, $P = 0.878$), it had a significant influence on the total larval survival of the clutch (from egg to diapause larvae) ($F_{12,107} = 2.46$, $P = 0.008$).

The propensity of females to remate was not affected by the male's previous number of matings ($F_{1,62} = 0.30$, $P = 0.588$), the time since the male's last mating ($F_{1,18} = 0.34$, $P = 0.568$), or the male's age ($F_{1,62} = 0.411$, $P = 0.52$). Males that mated females chose to remate with were not different from other males with respect to weight ($F_{1,52} = 1.16$, $P = 0.287$), total number of matings achieved ($F_{1,52} = 0.01$, $P = 0.911$), total number of offspring produced ($F_{1,52} = 0.07$, $P = 0.796$), offspring egg hatching rate ($F_{1,52} = 1.10$, $P = 0.299$), or offspring survival ($F_{1,52} = 1.40$, $P = 0.242$). However, they were on average younger than the other males present in the cage at the time of the mating ($F_{1,79} = 6.27$, $P = 0.014$).

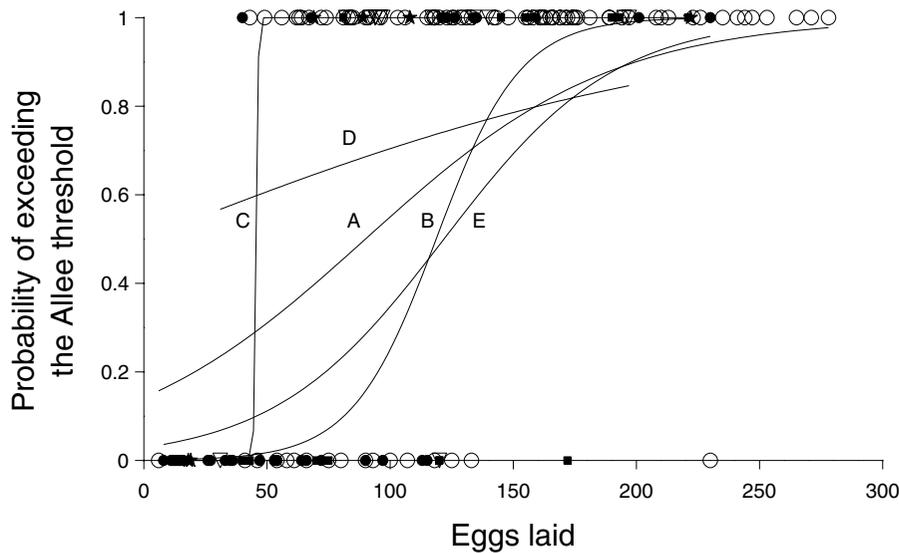


Figure 4. Logistic regressions for the probability of exceeding the Allee threshold at prediapause for clutches of type A (circles), B (small filled squares), C (stars), D (open triangles), and E (filled dots); see text for definitions of clutch type. The increase of probability with clutch size is highly significant ($P < 0.001$) for all types except clutches of mixed paternity (type D, $P = 0.16$, $n = 6$). However, the differences between the regressions are not significant in a mixed model (logistic regression) with female identity as a random factor. In particular, there is no significant difference between categories B (“first male of polyandrous females”) and C, D, or E (“later male”), either when C, D, and E were pooled to constitute one category, or when comparing separately with B: significance of the category was $P > 0.2$ in each case except for the comparison between B and C, in which case there was too little data to keep female identity as a random factor, and ignoring it produced a nonsignificant tendency ($P = 0.06$) that small clutches 100% sired by a later male exceed the Allee threshold more easily than equally small clutches sired by the first male.

The number of eggs a female laid was not affected by the male’s age ($F_{1,78} = 0.61$, $P = 0.44$) or the time elapsed since its last mating ($F_{1,25} = 1.57$, $P = 0.22$). Egg hatching rate and larval survival were not affected by the male’s age (egg hatching rate: $F_{1,56} = 0.08$, $P = 0.78$, larval survival: $F_{1,51} = 1.93$, $P = 0.28$), previous number of matings (egg hatching rate: $F_{1,56} = 0.32$, $P = 0.57$, larval survival: $F_{1,51} = 1.82$, $P = 0.18$), or time elapsed since last mating (egg hatching rate: $F_{1,20} = 1.13$, $P = 0.30$, larval survival: $F_{1,19} = 0.43$, $P = 0.52$).

Discussion

Within-generation bet hedging that is reducing the variance in the number of offspring to avoid having no or very few offspring in any given generation is theoretically difficult to maintain if it comes at a cost of not maximizing the expected number of offspring. We found that clutches of polyandrous females had a significantly lower variance than monandrous females in the number of offspring that survive to reach diapause. Thus, polyandrous females appeared to benefit of bet hedging. However, bet hedging did not appear to come at a cost of reduced total number of offspring surviving to diapause. Below, we discuss why *M. cinxia* can be particularly prone to experience advantages of within-generation bet hedging, without having to pay the associated cost.

The first reason why bet hedging can be important is the metapopulation structure of *M. cinxia* in the Åland Islands (Hanski

1999; Nieminen et al. 2004). Local populations are small, often extremely so: females disperse only after they have mated, and may singly found an entire new local population (Hanski 1999; Hanski et al. 1995). Ensuring that at least some offspring are viable through variance reduction is then a valid argument in favor of bet hedging (Yasui 1998). This can be achieved either by mating multiply before dispersal, or alternatively in the new patch if new males are encountered there. In this latter case the opportunity to reduce the variance arises after dispersal, but the same logic still applies, as long as the local populations remain small.

How strong is our evidence for bet hedging in *M. cinxia*? Of the two predictions and three conditions we derived for within-generation bet hedging, our data are in full agreement with both predictions and two of the conditions. Condition 3, that females should run a lower risk of all their clutches falling below the Allee threshold, was only supported in the sense of a nonsignificant trend.

The egg-laying pattern is particularly intriguing. Polyandrous females laid smaller clutches, and importantly, they did so already before they had mated twice—which is consistent with the idea that polyandry is a form of bet-hedging strategy that manifests itself already before the female has found another mate, rather than a chance event that occurs for some females, possibly controlled by male rather than female behavior. Consequently, we consider the alternative that the mating pattern is a result of male manipulation

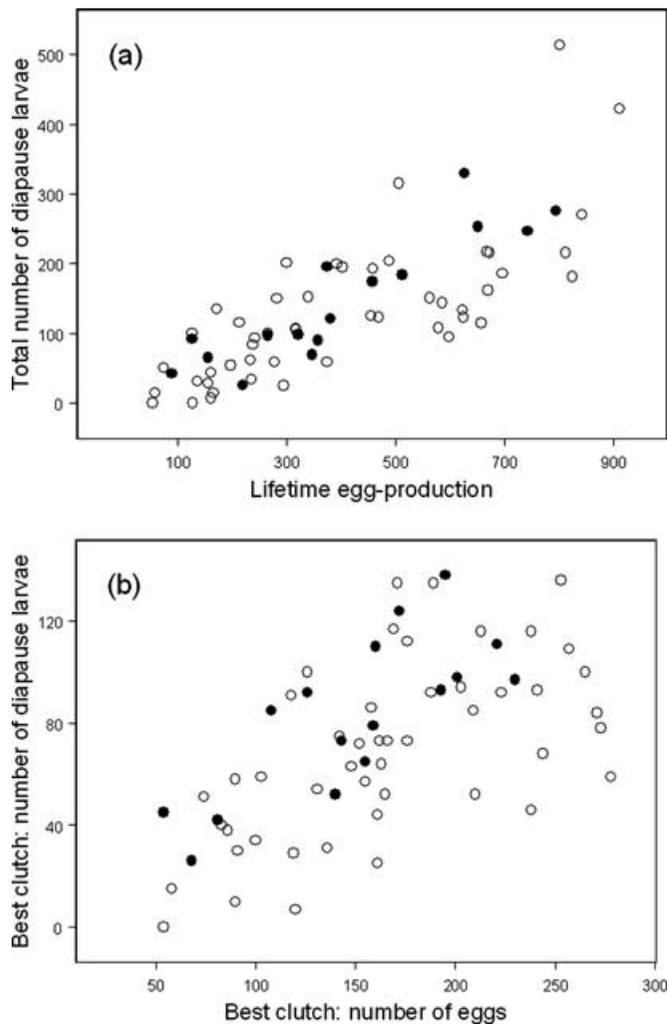


Figure 5. Offspring fitness (n) for monandrous and polyandrous females. a. Number of diapause larvae regressed against total number of eggs produced by each female. b. Fitness of each female's best clutch regressed against clutch size. Open circles: monandrous females; solid circles: polyandrous females.

(Arnqvist and Rowe 2005) unlikely, and we definitely detected no cost of multiple mating in terms of reduced life-time reproductive success or other measures of fitness. Another potential form of sexual conflict occurs when males prevent remating: in butterflies, mating plugs are widespread (Ehrlich and Ehrlich 1978) and male ejaculate often contains apyrene sperm that is known to influence female receptivity by filling the spermatheca and delaying female remating (Cook and Wedell 1999). *Melitaea cinxia* shows no obvious evidence of a mating plug (Wahlberg 1995), but we cannot exclude the possibility that males could influence female remating in more subtle ways. However, support for such a hypothesis was not found either, as male identity had no clear influence on the future mating behavior of the female.

Because bet-hedging individuals do not strive to maximize arithmetic mean fitness, they are often expected to pay a cost in

terms of this fitness measure (Hopper and Rosenheim 2003): indeed, theoretical results of bet hedging often phrase it as a trade-off between mean and variance of offspring numbers (Gillespie 1974, 1975, 1977; Proulx 2000). Therefore, all other factors being equal, multiply mating females should have a lower total number of surviving offspring (Fig. 3a). Our failure to find this cost indicate that there are additional factors that play a role in determining the fitness of bet hedging females. There is nothing in the definition of bet-hedging that prohibits it from co-occurring with other benefits of multiple mating. Among possible benefits that we cannot completely exclude is that polyandrous females gained additional fitness through a "trade-up" mechanism. Females can be particularly likely to compensate by remating with genetically superior (Hasselquist et al. 1996; Kempnaers et al. 1997; Pitcher et al. 2003) or more compatible males (Garner and Schmidt 2003; Masters et al. 2003) if their first mate is somehow found unsatisfactory. This could in principle explain the similar total number of surviving offspring between monandrous and polyandrous females (including a trend of more offspring for polyandrous females). Indeed, later sires showed a marginally significant tendency to produce better results, in terms of exceeding the Allee threshold, than first sires, although lack of power due to low sample size of confirmed paternity in later clutches warrants caution in applying this argument.

Direct benefits appear less likely as an alternative explanation, as polyandrous females did not lay more eggs. Although there is evidence that multiple mating in insects is often related to direct benefits, the effects found are typically very small (Fox 1993; Torres-Vila et al. 2004). In the closely related *Euphydryas editha*, variation in spermatophore size had no significant effect on female reproductive output (Jones et al. 1986) and spermatophores are unlikely to function as nuptial gifts in *M. cinxia* either (Boggs and Nieminen 2004). Females could also remate to ensure a sperm supply. Even though sperm production can be limited in males (Olsson et al. 1997), *M. cinxia* males do not seem to be sperm limited, even after several matings or when the time between matings is very short.

Regarding condition 3 which, as must be stressed, was not supported with full statistical significance, it is also worth pointing out a feature of the experimental setup that causes it to underestimate the variance in male compatibility and hence the importance of bet hedging. The butterflies originated from large, well-connected populations, and were thus not expected to be inbred. In natural conditions, there is high inbreeding depression in small populations, and high relatedness between the parents causes an important and significant decrease in offspring egg-hatching rate and survival (A. Sarhan and S. Haikola, unpubl. ms.). Previous results show that relatedness between the parents is much more important in determining offspring fitness than either parent's heterozygosity (A. Sarhan and S. Haikola, unpubl.

ms.). If the indiscriminate female mating behavior extends to accepting related males, as shown in Haikola et al. (2004), and often expected to be adaptive when males are encountered sequentially (Kokko and Ots 2006), the variance in offspring fitness would be much higher for monandrous females, and the difference between monandrous and polyandrous females would be similarly magnified.

Finally, a particular feature in the biology of *M. cinxia* means that the prospects for finding a significant evolutionary advantage of bet hedging could be much elevated for this species in the particular case of mixed paternity in a single clutch of eggs. Overwintering in communal nests means that it is very important for a female to have at least one clutch that has at least 25 larvae at prediapause. The nonindependence of larval survival means that mixing paternity can be advantageous for guaranteeing that at least some offspring in each clutch are highly viable, and the clutch as a whole therefore exceeds the required threshold. Unfortunately, this specific benefit of bet hedging was difficult to test in our dataset that yielded few clutches of mixed paternity. These appear to perform very well in exceeding the Allee threshold even at small clutch sizes (category D in Fig. 4), but statistical significance is lacking. How likely this factor is to influence bet-hedging strategies in nature will depend on how often females have control over paternity, above the usual pattern of last male sperm precedence (Bonduriansky 2001; Eady et al. 2004).

Finally, it must be kept in mind that there are multiple explanations of multiple mating, which makes it impossible to falsify all alternatives with a single study. Nevertheless, both the intriguing egg-laying pattern of polyandrous females and the resulting lowered variance in their life-time reproductive success suggest that within-generation bet hedging is in this case an unusually likely candidate for explaining the occurrence of polyandry as a successful evolutionary strategy. This obviously begs the question why polyandry has not spread to fixation: theory shows that in a metapopulation with local density regulation selection against demographic stochasticity can be strong even though the entire population is large (Proulx 2000). There are several potential explanations, one of which is the need to produce many clutches which necessitates a sufficiently long life span. Whether such a cost is significant in nature remains to be tested. Our cage setup did not detect such a cost at the level of life-time fecundity, even though predation was possible in the cage. Birds were obviously excluded from the cage setup, but checkerspot sequestrate chemical compounds and they rarely suffer predation from birds (Nieminen et al. 2004). Predation by spiders and predatory insects is a much more important cause of mortality in *M. cinxia* (Nieminen et al. 2004), and spiders were very abundant in the cage (M. Saastamoinen, pers. comm.).

Alternative possibilities include the metapopulation structure of *M. cinxia* that consists of patches that differ in connectivity. This

has been found to create spatial variation in other traits such as dispersal and the associated fecundity (Haag et al. 2005; Hanski et al. 2006), and mating strategies could also conceivably differ between well-connected populations and small, isolated ones. The conditions of bet hedging are generally better met in the latter, which creates interesting possibilities of spatially varying selection.

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