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Molecular evidence of post-copulatory inbreeding avoidance in the field cricket *Gryllus bimaculatus*

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Female promiscuity has broad implications for individual behaviour, population genetics and even speciation. In the field cricket *Gryllus bimaculatus*, females will mate with almost any male presented to them, despite receiving no recorded direct benefits. Previous studies have shown that female crickets can benefit from polyandry through increased hatching success of their eggs. There is evidence that this effect is driven by the potential of polyandrous females to avoid fertilizing eggs with sperm from genetically incompatible males. We provide direct evidence supporting the hypothesis that polyandry is a mechanism to avoid genetic incompatibilities resulting from inbreeding. Using microsatellite markers we examined patterns of paternity in an experiment where each female mated with both a related and an unrelated male in either order. Overall, unrelated males were more successful in gaining paternity than were related males, but this effect was driven by a much greater success of unrelated males when they were the first to mate.

Keywords: cryptic female choice; genetic incompatibility; Orthoptera; paternity; sperm competition; sexual selection

1. INTRODUCTION

Polyandry is widespread and is increasingly recognized as behaviour with broad implications (for a review see Jennions & Petrie 2000). Molecular techniques have revealed multiple paternity in a range of taxa (Schwartz *et al.* 1989; Dunn & Lifjeld 1994; Schenk & Kovacs 1995; Moore & Ball 2002), including species previously thought to be monogamous (Petrie *et al.* 1998). Mating rates clearly have ramifications for sexual selection, providing scope for sexual conflict (Parker 1979) and sperm competition (Gowaty 1994; Birkhead & Møller 1998), with implications not just for individual behaviour, but also for higher-level processes such as speciation (Arnqvist & Nilsson 2000; but see also Gage *et al.* 2002). As mating inevitably carries costs (Daly 1978; Watson *et al.* 1998), the prevalence of polyandry suggests that females may mate repeatedly for reasons other than a simple requirement to acquire sufficient sperm to fertilize their eggs. Direct benefits of polyandry, such as nuptial gifts (Gwynne 1984; Vahed 1998; Reinhold 1999) or increased paternal care (Ihara 2002), have clearly played a role in the evolution and maintenance of polyandry in some species. However, other species have no such obvious direct benefits, raising the possibility that females can profit from polyandry through genetic benefits that increase the fitness of their offspring. In a number of species, offspring viability is increased when females have a greater number of mates (Madsen *et al.* 1992; Gowaty 1994; Olsson *et al.* 1994; Keil & Sachser 1998; Tregenza & Wedell 1998; Kempenaers *et al.* 1999; Newcomer *et al.* 1999). Suggested genetic benefits include 'trading up' to better-quality mates (Kempenaers *et al.* 1992), bet hedging against poor-quality mates (Watson 1991) and increased genetic variability of offspring (Baer & Schmid-Hempel 1999).

Recent attention has focused in particular on the suggestions that offspring fitness may depend on the compatibility of the parents' genomes (Zeh & Zeh 1996) and that polyandrous females may be able to bias paternity or investment in offspring in favour of more compatible mates. A number of potential sources of genetic incompatibility have been proposed (Zeh & Zeh 1997), although it has been argued that, of these, costs arising as a result of processes associated with inbreeding (Pusey & Wolf 1996) are likely to prove to be by far the most widespread (Tregenza & Wedell 2000).

Females of the field cricket *Gryllus bimaculatus* have been shown to benefit from polyandry through increased hatching success of their eggs (Tregenza & Wedell 1998). This effect is most simply explained by the hypothesis that polyandrous females can avoid using sperm from incompatible males, but the source of genetic incompatibility has not been known. Previous work (Tregenza & Wedell 2002) has shown that females will mate readily with any courting male placed in close proximity to them, including full siblings. There are several potential explanations for this behaviour: (i) females may only rarely encounter siblings in the wild, removing any selection pressure to avoid mating with them; (ii) mating with a sibling may be better than remaining unmated, so females may sometimes benefit from incestuous matings; (iii) it may be less costly to mate with a brother than to endure his harassment; (iv) females may need close contact or even to mate to identify and so to discriminate against siblings. In a recent study in which the potential role of inbreeding depression was investigated, it was found that females mating with two sibling males had lower egg-hatching success than those mating with two non-siblings. However, when females mated with both a sibling and a non-sibling male they had the same hatching success as females mated with two unrelated males rather than an intermediate hatching success, as might be expected if paternity is unbiased (Tregenza & Wedell 2002). The observation that inbreeding depression was not evident when females mate with at least one

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unrelated male highlights an intriguing question—can females discriminate against sperm from siblings?

2. MATERIAL AND METHODS

We used parental and offspring crickets retained from a study by Tregenza & Wedell (2002). Rearing conditions were 29 °C, an 18 L : 6 D photoperiod and free provision of food and water. All individuals were F_2 descendants of gravid females collected from the wild in Gaborone, Botswana. One virgin F_1 female from each parental female was mated to a single male from another family to create a set of unrelated full-sibling families. Experimental individuals were collected from families as late-instar nymphs to ensure virginity and were isolated in separate pots. All females were virgins. All males had mated with an unrelated female on the previous day. The experiment was arranged in blocks of four females and two males from one family and two sibling males from another family. Within each block, females were mated to two siblings (SS), to two non-related males (NN), to a sibling then a non-related male (SN), or vice versa (NS). Four males were used in each block so that each female's second mate was a male that had mated in the previous hour. Previous studies have shown that males do not suffer from sperm depletion between first and second spermatophores (Simmons 1986; Simmons 1987*b*). We checked to confirm that males had a spermatophore ready for transfer before being introduced to a female. Almost all pairs mated within 10 min, but if no mating occurred within an hour, the female was replaced with one of her sisters. The male was left with the female for at least 45 min after mating, preventing the female from removing the spermatophore. In the females whose offspring were genotyped for this study the interval between matings was on average 84.7 ± 4.9 min (mean ± s.e.m.) with a maximum of 149 min. All second males had previously been used in the role of first mate.

After mating, females were provided with fine wet sand, which was kept moist at all times. After 3 days the sand was sieved to remove the eggs. The eggs were counted, placed on a wet cotton-wool pad in a 9 cm diameter Petri dish and maintained under the same conditions as the adults. Eggs were checked daily for hatching until 7 days after the last emergence. Adults and hatchlings were kept at −20 °C until DNA extraction. Adult DNA was extracted using a phenol–chloroform method (Sambrook *et al.* 1989). Nymph DNA was extracted using a modified salting-out method for whole insects (Strassmann *et al.* 1999). In total, DNA was extracted from 40 crosses, but three were later disregarded because a parental genotype could not be resolved, or because fewer than 15 nymphs could be genotyped after at least three attempts. DNA quality and quantity were assessed on 2% agarose gels stained with ethidium bromide.

Adults were genotyped using three polymorphic loci: *Gbim04* (11 alleles from 15 individuals), *Gbim14* (14 alleles from 15 individuals) and *Gbim15* (eight alleles from 20 individuals) (Dawson *et al.* 2003). The PCR profile was 94 °C for 4 min (one cycle), 94 °C for 30 s, X °C for 30 s and 72 °C for 30 s (35 cycles) and 72 °C for 10 min (one cycle), where $X = 65$ °C for *Gbim04* and *Gbim15*, and 57 °C for *Gbim14*. Each 10 µl PCR mixture contained 0.1–10 ng of genomic DNA, 1 µmol of each primer, 0.2 mmol of each dNTP and 0.25 units of *Taq* DNA polymerase (ThermoprimePlus, ABGene, Epsom, Surrey, UK) in the manufacturer's buffer (final concentrations were 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl, pH 9.0 and 0.01% (w/v) Tween), including 1.5 mM $MgCl_2$. PCR amplification was performed in a Hybaid Touchdown thermal cycler (Thermo Hybaid, Ashford, Middlesex, UK). Products were diluted with water (*Gbim04* 1 : 14, *Gbim14* 1 : 4.5, *Gbim15* 1 : 24) and were multiplexed for loading on gels (1.5 µl of each diluted product mixed, then 1.5 µl of the multiplex added to 2 µl of loading buffer).

Some individuals had been observed to be homozygotes when initially PCR-amplified but were found to be heterozygotes under different conditions. To avoid uncertainty as to the alleles being amplified, putative homozygotes from families where the female did not share one or both alleles with either male were PCR-amplified seven times in accordance with the protocol described by Taberlet *et al.* (1996) to reduce the chance of missing alleles. This affected 13 families, with a mean of 4.4 individuals per family amplified and run seven times.

3. RESULTS

Paternity was assigned by the presence of unique alleles from the putative fathers, 22 families using one locus, 10 using two loci and five using three loci. Out of the 40 families where informative loci were identified, 37 yielded paternities for between 15 and 20 offspring (mean ± s.e.m. = 18.5 ± 0.25), 19 in the NS treatment and 18 in the SN treatment. The remaining three families were not included either because a parent was missing or because fewer than 15 offspring could be genotyped.

(a) *Paternity, relatedness and mating order*

The mean paternity of the non-related male (P_N) was 0.90 in the NS group and 0.45 in the SN group. However, because only offspring that hatched were genotyped, a correction must be applied to allow for the bias arising from the higher hatching success of eggs fertilized by sperm from non-related males. In the study by Tregenza & Wedell (2002), the hatching success of females mated to two non-related males was 1.5 times that of females mated to two of her siblings. Hence, where a female was mated to one non-related and one sibling male, even if the fertilization successes of male types were equal, the offspring that survived to hatching would be 1.5 times more likely to be sired by the non-related male. All paternity estimates were adjusted accordingly, a procedure that is conservative in relation to the hypothesis that females may avoid using sperm from related males. There was no effect of interval between first and second mating, on corrected P_2 (the proportion of offspring sired by the second male to mate) in either group (NS group: $n = 19$, Spearman's $r = -0.17$, $p = 0.5$; SN group: $n = 18$, $r = -0.06$, $p = 0.8$).

Using the corrected data, the mean (± standard error) paternity of the non-related male (P_N) was 0.84 (± 0.039) in the NS group and 0.38 (± 0.066) in the SN group. The proportion of offspring sired by the non-related male for each female can be seen in figure 1. In 24 out of the 37 families the non-related male sired more offspring than the sibling male (19 out of 19 in the NS group, 5 out of 18 in the SN group). Each family was tested individually for deviation from the hypothesis of equal paternity for each male ($P_2 = 0.5$) predicted by free sperm mixing. In the NS group, 13 out of 19 families (11 out of 19 after Bonferroni correction) differed from a 1 : 1 ratio of paternity for the two males. In the SN group, 11 out of 18 families differed significantly from 1 : 1. Out of these 11 families, in nine (four after Bonferroni correction) the

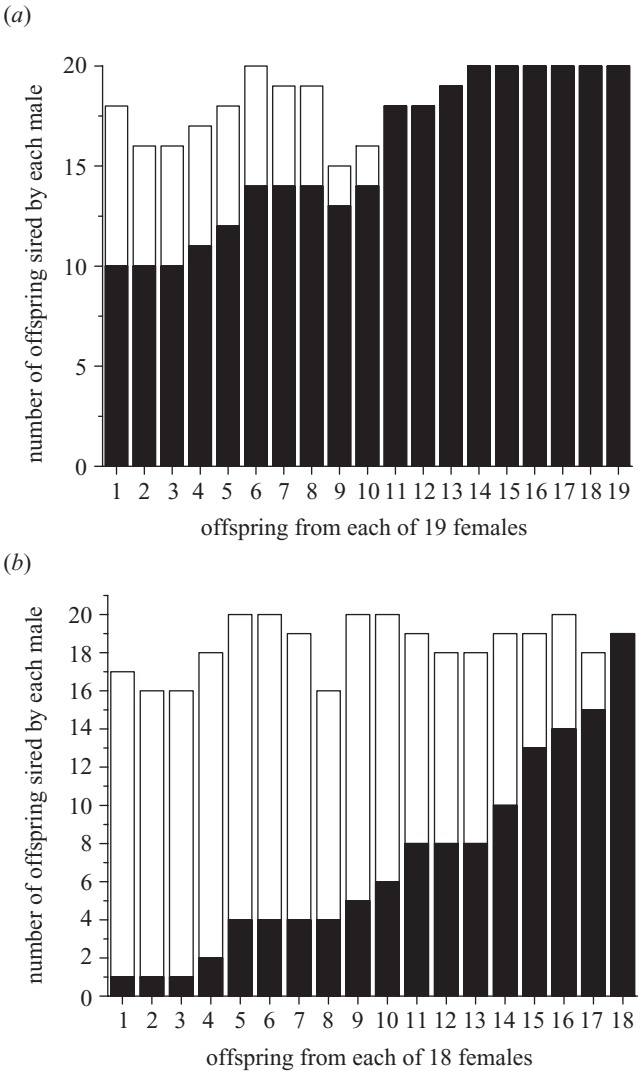

Figure 1. Number of offspring assigned to each putative father, where either (*a*) the non-related male (N; filled bars) or (*b*) the sibling male (S; open bars) mated first. Actual numbers of offspring per male are shown before correction for overestimation of offspring from non-related fathers in hatched offspring. After correction, these numbers were tested against free sperm mixing ($P_2 = 0.5$) using χ^2-tests. In (*a*), paternities in clutches 7 to 19 differ significantly from $P_2 = 0.5$ ($p < 0.05$), all in favour of the non-related male. In (*b*), clutches 1 to 9 and 17 and 18 differ significantly from $P_2 = 0.5$ ($p < 0.05$), nine in favour of the sibling and two in favour of the non-related male.

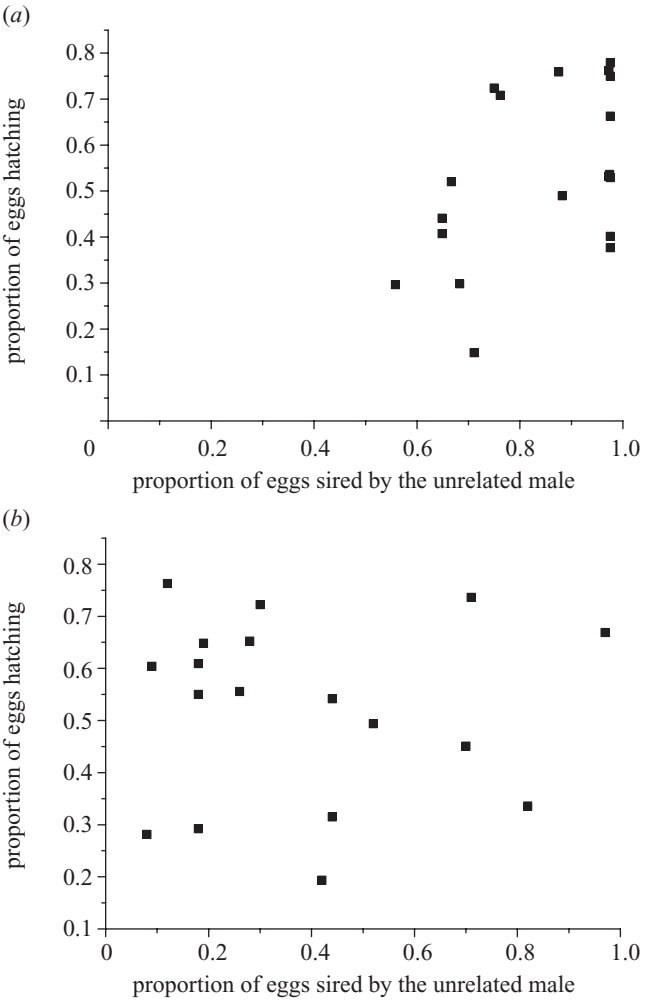

Figure 2. Hatching success and paternity by the non-related male in (*a*) the NS group (non-related male mates first) and (*b*) the SN group. Paternity has been corrected for overestimation of offspring from non-related fathers in hatched offspring. The positive trend in the NS group is not significant ($n = 19$, $r = 0.4$, $p = 0.08$) and there is no pattern in the SN group ($n = 18$, $r = 0.012$, $p = 0.961$).

sibling male gained a significantly greater share of paternity, and in two (one after Bonferroni correction) the non-related male gained a greater than expected share of paternity. These data could not be pooled within treatments for analysis owing to differences in the pattern of paternity between families within treatments (heterogeneity χ^2-test, NS: $\chi^2 = 38$, $p = 0.007$, d.f. = 18; SN: $\chi^2 = 107$, $p < 0.001$, d.f. = 17).

(**b**) *Paternity and hatching success*

If offspring sired by non-related males have higher embryonic viability, the hatching success of females mating with both a related and a non-related male may be higher where the non-related male has a higher share of paternity (assuming equal costs of inbreeding between crosses—see § 4). We did not find such a relationship between P_N and hatching success. In the NS group there was a trend in the predicted direction (figure 2*a*), but this was not significant (Spearman's rank correlation: $n = 19$, $r = 0.42$, $p = 0.076$), and no pattern can be seen in the SN group ($n = 18$, $r = 0.012$, $p = 0.96$).

(**c**) *Comparisons between sisters*

In the study carried out by Tregenza & Wedell (2002), groups of four sisters were mated to each combination of males (NN, SS, NS and SN), so that within replicates one sister was in the NS group (mating with a non-sibling followed by a sibling) and one in the SN group (mating with a sibling followed by a non-sibling), but not all reciprocals could be genotyped. There were 13 cases in which both sisters were genotyped. The relationship between sisters was explored, to ascertain whether there was a correlation in P_2 or P_N between sisters. No such relationship was found in either case (Spearman's rank correlation: P_2: $n = 13$, $r = -0.18$, $p = 0.56$; P_N: $n = 13$, $r = 0.18$, $p = 0.56$).

4. DISCUSSION

This study provides, to our knowledge, the first marker-based paternity analysis in *G. bimaculatus*. Although the data cannot be pooled for analysis, it is clear that paternity deviates from the 50 : 50 predicted by a free sperm-mixing model. Our study does not allow us to estimate P_2 independently of male relatedness. However, there is substantial evidence from irradiated male studies by Simmons (1987*a*) and by Morrow & Gage (2001) that paternity is not biased in relation to mating order in this species. The average P_2 of 0.45 in males given an equal number of matings, reported by Simmons (1987*b*), and the figure of 0.45 in males in a line selected for sperm length close to the population mean, reported by Morrow & Gage (2001), are comparable with the figure of 0.38 in the SN category in the present study. The marginally higher P_2 value found by Simmons (1987*b*) and by Morrow & Gage (2001) may be associated with differences in time between matings. If the time between matings is not long enough for the first male's sperm to be transferred into the spermatheca, this could act as a barrier to the second male's sperm. Times between matings tend to be quoted as those anticipated in the experimental design, rather than exact values measured, making it difficult to compare studies. When we examined the actual recorded times in the study by Tregenza & Wedell (2002) we found that the mean mating interval was greater than had originally been stated. However, there was no relationship between mating interval and P_2, suggesting that intervals are sufficiently long to ensure full sperm transfer.

It is possible that some offspring were falsely scored as homozygotes, inflating the number of offspring sired by the sibling, though strenuous attempts to avoid this possibility were made by repeated PCR amplification of dubious individuals. This was not done for all homozygotes, only those that were suspected to be heterozygotes, for example because the female shared only one allele with either male.

(a) *Relatedness*

Relatedness of parents has a substantial effect on paternity. If the observed difference in paternity between related and unrelated males was entirely a result of mating order, P_2 would be similar in both groups. However, we found that the non-related male achieves a dramatically higher paternity when mating first than would be expected if there was no effect of relatedness. By contrast, when the non-related male is the second to mate he does not achieve higher paternity than the sibling male. This is not a result of offspring sired by non-related males being more likely to hatch, as this was taken into account in the analysis. A possible explanation for this pattern is that virgin females store sperm from both related and non-related males, but when mating for a second time, only females that have previously mated with a sibling store a significant number of sperm from their new mate. This does not require the female actively to choose individual sperm within the spermatheca, but simply a mechanism by which females either do not transfer the ejaculate to the spermatheca or expel the ejaculate from related males.

The observed pattern of paternity in relation to mating order has similarities to that predicted by the 'trade-up' hypothesis (Halliday 1983), which is the idea that females may mate with the first male encountered to ensure fertilization, but subsequently mate preferentially with males of higher genetic quality. However, in our case, it appears that, rather than trading up, female crickets avoid trading down by limiting their use of sperm from related males if they already have sperm from unrelated males. Bateman *et al.* (2001) found that previously mated female crickets discriminate between males on the basis of size, whereas naive females do not, suggesting that female field crickets may trade-up (or possibly avoid trading down) using pre-mating choice. The only examination of the trade-up hypothesis to incorporate post-mating processes is a study of paternity in doubly mated guppies (*Poecilia reticulata*). Pitcher *et al.* (2003) found that both mating order and male coloration affected paternity. However, in contrast to our study, if the data published by Pitcher *et al.* (2003) are compared with expected levels of sperm precedence arising from mating order (using data from Evans & Magurran 2001), it appears that colourful males have an advantage over drab males in the role of both first and second mate.

An alternative explanation for the observed mate-order effect could be that females learn to discriminate against kin. Simmons (1989) provides evidence that females become better at recognizing kin with increased exposure to unrelated males. If this is the case, females initially presented with a non-related mate may be better able to discriminate against their brothers and hence bias paternity towards the non-related male than are females mated to the sibling male first. This type of process could also explain the observation of Bateman *et al.* (2001) that naive females are less discriminatory over the size of their mates: they may need to learn about the size distribution of potential partners.

Why females should use post-copulatory inbreeding avoidance rather than simply avoid mating with relatives remains to be elucidated. The two processes are not mutually exclusive, and may be complementary. Close contact or even copulation may be necessary to detect cues to relatedness such as cuticular pheromones or chemical cues from the sperm itself. Additionally, it may be energetically less expensive to exercise post-copulatory mate choice, since male harassment may be reduced.

(b) *Hatching success*

Even without invoking a sperm-choice mechanism, the relationship between the proportion of offspring sired by non-related males (P_N) and hatching success might be predicted to be either positive or negative depending on the influence of inbreeding depression. If inbreeding depression is constant between crosses but males differ in fertilization success independently of relatedness, a positive relationship between P_N and hatching success will be observed. This is because when the male with greater success in sperm competition happens to be the unrelated male, more offspring will hatch and a larger proportion of them will be sired by the unrelated male. Alternatively, if the effect of inbreeding on hatching success differs between crosses, then those with higher levels of egg mortality as a result of inbreeding will have a lower overall hatching success, but a greater proportion of those hatching will be from the non-related male. Hence there will be a negative correlation between overall hatching and the

proportion sired by the non-related male. Given that inbreeding depression has a genetic basis, its effects are likely to vary between crosses. In the study carried out by Tregenza & Wedell (2002) the hatching success of females mated to two siblings was normally distributed (Kolmogorov–Smirnov test = 0.131, $n = 112$, $p = 0.2$). This is evidence for inbreeding depression varying between crosses, but it does not indicate whether hatching success also varies between sisters and hence does not allow us to correct the under-representation of paternity by the sibling male in the hatched offspring for each family individually. To determine whether more incompatible males are less successful in sperm competition would require determination of paternity before mortality arising from inbreeding occurs. Our data do not show a clear pattern, although the positive relationship between sperm-competitive success and hatching success in figure 2*a* is the opposite to that predicted if females bias sperm use more severely when genetic incompatibilities are more severe.

The difference in paternity bias according to male mating order raises the question of why the NS treatment in the study by Tregenza & Wedell (2002) did not result in higher hatching success than the SN treatment. One possible answer is that there are multiple factors affecting hatching success; for example, if there are 'good gene' effects acting equally across treatments, these may reduce the power of the experiment to detect effects arising from relatedness alone.

(c) *Comparisons between sisters*

We found no evidence that sisters have correlated levels of P_2. This suggests that physical traits such as spermathecal size that could be similar between sisters are not an important determinant of paternity. We also found no evidence for differences between pairs of sisters differ in their ability to discriminate between siblings and unrelated males. This effect may have been apparent if, in some blocks, females shared grandparents with the non-related males and hence could not discriminate between mates as easily as could the females in other blocks.

In conclusion, the results of our study support the hypothesis that females mating with both related and unrelated males avoid costs of inbreeding through a bias in paternity in favour of unrelated males. Future work will cover the mechanisms that create this bias and the extent to which wild females are exposed to the risks of inbreeding. Female multiple mating and costs of mating with relatives are both extremely common across taxa. Studies on the possible existence of post-copulatory inbreeding avoidance in other groups would be valuable.

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